Characterization of peripheral and lesional single B cell autoreactivity in patients with Sjögren's syndrome and rheumatoid arthritis

By

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DECLARATION

I declare that the materials contained in this thesis have not been used in any other submission for an academic award. All the sources of investigation have been duly acknowledged and the thesis does not exceed 100.000 words.

The studies presented in this thesis were conducted in the department of Experimental Medicine and Rheumatology at the William Harvey Research Institute, Queen Mary University, London.

The thesis has been written by me and the work presented in this manuscript is the result of my investigation with the following exceptions:

Dr Michele Bombardieri actively contributed in the project development and writing of the paper derived from this Thesis.

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ABSTRACT

Sjögren's syndrome (SS) and rheumatoid arthritis (RA) are characterised by breach of self-tolerance with high affinity circulating autoantibodies and peripheral B cell disturbances in the naïve and memory B cell compartments. In addition, both SS and RA develop functional ectopic B cell follicles in the respective target organs, i.e. the salivary glands and the joint synovium, whereby autoreactive B cell undergo antigen selection and affinity maturation. However, the exact stage at which errors in B cell tolerance checkpoints accumulate is unknown.

In this PhD project, I amplified and sequenced Ig VH and VL gene transcripts from single B cells which were FACS sorted either from the peripheral blood of SS patients or from the RA synovium. Healthy donors (HD) were used as controls. Subsequently, I cloned and expressed recombinant monoclonal antibodies displaying identical antigenic specificity of the original B cells. Finally, I tested the poly- and autoreactivity profile of these antibodies against SS and RA-associated autoantigens.

In SS, I analysed 353 VH and 293 VL sequences and obtained 114 recombinant antibodies from circulating naïve (n=66) and memory (n=48) B cells of 4 SS patients and compared their autoreactive and polyreactive profile to 45 naïve clones from 2 HD. Analysis of the VH and VL gene usage showed no significant differences between SS and HD. Conversely, I observed accumulation of circulating autoreactive naïve B cells in SS as demonstrated by Hep-2 cells, ENA, Ro/SSA and/or La/SSB reactivity. The elevated frequency of autoreactive naïve B cells in the circulation of SS patients supports the existence of early defects in B cell tolerance checkpoints in this condition.

In RA, I analysed the Ig gene repertoire and the VH gene somatic mutation rate of 139 VH and 175 VL sequences of synovial CD19+ B cells which demonstrated evidence of antigen selection and hypermutated alpha>gamma>mu VH chains with presence of intra-synovial clonal diversification. Recombinant antibodies from synovial B cell clones were then screened for reactivity towards citrullinated antigens with a plan for a wider analysis using autoantigen microarrays.

Overall, these results highlighted the existence of B cell abnormalities and loss of tolerance for self-antigens both in the peripheral and/or lesional compartment of SS and RA. Further analysis of the fine specificity and pathogenicity of recombinant antibodies from autoreactive B cells will be invaluable in order to dissect the mechanisms and the antigens driving the development and the persistence of autoimmunity in RA and SS.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AID	Activation induced cytidine deaminase
AMA	Anti-mitochondrial antibody
ANA	Anti-nuclear antibody
anti-SSA	anti-Sjögren's Syndrome A
anti-SSB	anti-Sjögren's Syndrome B
APC	Antigen presenting cell
BAFF	B-cell activating factor of the TNF family
BCL-2	B-cell lymphoma 2
BCL-6	B-cell lymphoma 6
BCR	B-cell receptor
Blimp1	B lymphocyte induced maturation protein 1
CCL	Chemokine (C-C motif) ligand
CD	Cluster differentiation
CDR	Complementary determining region
CSR	Class switch recombination
CTLA	Cytotoxic T-lymphocyte antigen
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
DMARD	Disease-modifying antirheumatic drug
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EBER	Epstein-Barr virus-encoded small RNA
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELS	Ectopic lymphoid structure
ENA	Extractable nuclear antigen
EULAR	European League Against Rheumatism
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDC	Follicular dendritic cell
FR	Framework region
GC	Germinal center
HCV	Hepatitis C virus
Hep-2	Human epithelial type 2
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

HMGB1	High-mobility group protein B1
HRP	Horseradish Peroxidase
ICAM	Intercellular adhesion molecule
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LESA	Lymphoepithelial sialoadenitis
LN	Lymph node
LPS	Lipopolysaccharide
LT	Lymphotoxin
M3R	Muscarinic acetylcholine receptor 3
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MTX	Methotrexate
NF-κB	Nuclear factor κB
NHEJ	Non-homologous end joining
NOD	Non-obese diabetic
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC	Plasma cell
PCR	Polymerase chain reaction
PNAd	Peripheral node addressing
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid arthritis
RAG	Recombination activating gene
RANK	Receptor activator of nuclear factor κ B
RF	Rheumatoid factor
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RSS	Recombination signal sequence
RTX	Rituximab
SCID	Severe combined immunodeficiency
Scl-70	Scleroderma-70
SG	Salivary gland
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
Sm	Smith
SS	Sjögren's syndrome
T1D	Type 1 diabetes
TdT	Terminal deoxynucleotidyl transferase
TLR	Toll-like receptor

TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
VA	Virus associated
VCAM	Vascular cell adhesion molecule

LIST OF CD ANTIGENS RELEVANT TO THIS THESIS

CD antigen	Cellular expression	Molecular weight (kDa)	Functions
CD3 (T3)	Thymocytes, T cells	γ: 25-28 δ: 20 ε: 20	Associated with the T-cell antigen receptor. Required for cell-surface expression of and signal transduction by the TCR.
CD4 (T4,L3T4)	Th1 and Th2 T cells, monocytes, macrophages	55	Co-receptor for MHC class II molecules.
CD5	Thymocytes, T cells, subset of B cells	67	
CD10	B and T cells precursor, bone marrow stromal cells	100	Zinc metalloproteinase, marker for pre-B acute lymphatic leukemia (ALL).
CD14	Myelomonocytic cells	53-55	Receptor for complex of LPS and LPS binding protein.
CD19	B cells	95	Forms complex with CD21 (CR2) and CD81 (TAPA-1); co-receptor for B cells.
CD20	B cells	33-37	Oligomers of CD20 may form a calcium channel; possible role in regulating B cell activation.
CD21 (CR2)	Mature B cells; follicular dendritic cells	45	Receptor for complement component C3d, Epstein-Barr virus.
CD23 (FcεRII)	Mature B cells, activated macrophages, eosinophils, follicular dendritic cells, platelets	45	Low-affinity receptor for IgE; regulates IgE synthesis; ligand for CD19/CD21/CD81 co-receptor.
CD27	T cells, NK cells, some B cells	55	Binds CD70; co-stimulator for T and B cells.
CD28	T cell subsets, activated B cells	44	Activation of naïve T cells, receptor for co-stimulatory signal binds CD80 and CD86.
CD38	Early B and T cells, activated T cells, germinal center B cells, plasma cells	45	Augments B cell proliferation.

CD antigen	Cellular expression	Molecular weight (kDa)	Functions
CD40	B cells, macrophages, dendritic cells, basal epithelial cells	48	Binds CD40L; receptor for co-stimulatory signal for B cells, promotes growth, differentiation, and isotype switching of B cells, and cytokine production by macrophages and dendritic cells.
CD44	Leukocytes	80-95	Mediates adhesion of leukocytes.
CD45 (B220)	All hematopoietic cells	180-240	Tyrosine phosphatase, augments signalling through antigen receptor of B and T cells; multiple isoforms.
CD68	Monocytes, macrophages, neutrophils, basophils	110	Unknown
CD70	Activated T and B cells and macrophages	75,95,170	Ligand for CD27; may function in co-stimulation of B and T cells.
CD77	Germinal center B cells		Cross-linking induces apoptosis.
CD81	Lymphocytes	26	Associated with CD19, CD21 to form B cell co-receptor.
CD80 (B7.1)	B cell subset	60	Co-stimulator, ligand for CD28 and CTLA-4.
CD86	Monocytes, activated B cells, dendritic cells	80	Ligand for CD28 and CTLA-4.
CD138 (Syndecan-1)	B cells		Heparin sulphate proteoglycan binds collagen type I

Source: Janeway's Immunobiology, 8th Edition

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Chapter 1 | Introduction

1.1 Development of antibody-producing B cells and B cell self-tolerance in humans.

1.1.1 Introduction.

10-15% of human peripheral blood lymphocytes, 20-30% of lymph node cells, 50% of splenic lymphocytes, and around 10% of bone marrow lymphocytes are constituted by mature B cells. Each B cell expresses on its surface a unique intramembrane immunoglobulin (Ig) molecule, referred as B cell receptor (BCR) capable to bind to a specific antigen. Unlike T cells which can recognize only processed antigens expressed on the MHC class I and class II molecules on antigen presenting cells (APCs), B cells are able to recognize unprocessed native antigens via antigen binding to B cell surface Ig receptors. The primary function of B cells is to produce antibodies but they can also act as APCs. Moreover, B cells are involved in T cell activation, anergy, and differentiation as well as in the expansion of T cells [1]. B cells can also regulate follicular dendritic cell differentiation and organization of lymphatic structures [1]. Finally, B cells can produce pro-inflammatory cytokines (e.g., tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)) and anti-inflammatory cytokines such as IL-10 [1].

1.1.2 B cell development before antigen encounter.

B cells arise from stem cells in the bone marrow and they progress through several stages of maturation before entering the peripheral compartments – primarily lymph nodes and spleen, where they can differentiate into memory B and plasma cells which can finally migrate back to the bone marrow as long lived plasma cells (**Figure 1.1**) [2].

The earliest B cell precursors are **pre-pro-B cells** which have a low expression of the recombination activation genes (RAG-1 and RAG-2), involved in recombination process of the variable, diversity (only for the heavy chain), and joining regions or V(D)J, and they do not express elements of the BCR [3]. Pre-pro-B cells differentiate into **pro-B cells** which express a precursor form of the BCR formed by Igα, Igβ and calnexin (pro-BCR). At this stage of B cell development, V(D)J recombination starts at the Ig heavy (H) locus [3] leading to the synthesis of a productive Igμ chain. Ig gene recombination starts with D to JH segment rearrangements followed by VH and D-JH segment rearrangements [4]. This process requires the cytokine IL-7, expressed by several type of stromal cells in the bone marrow, the transcription factors Pax5 and YY1, and high levels of Rag-1/Rag-2 proteins [4]. Once a productive rearrangement is made, pro-B cells differentiate into **pre-B cells** which have to rearrange their light chain loci. The pre-BCR is formed by the transmembrane form of Igμ (mIgμ), the surrogate light chains (λ5 and VpreB), Igα and Igβ and its main functions are to start clonal expansion in a IL-7 independent way, heavy chain allelic exclusion, and further differentiation. Allelic exclusion means that after a successful rearrangement of one IgH allele, the other allele is inhibited from going through further rearrangements. After clonal expansion, pre-B cells stop in G1 phase, they become smaller and start Igκ locus rearrangement with the final expression of the IgM BCR [4]. At this stage pre-B cells differentiate into **immature B cells** which are the first B cells to express surface BCR. They express IgM but little or no surface IgD [4]. Immature B cells are particularly susceptible to BCR-induced apoptosis or **deletion**, compared to mature B cells. Deletion is one of the three main tolerance mechanisms used by the immune system

to reduce self-reactive B cells during development. **Anergy** is the second mechanism by which self-tolerance is maintained in immature B cells. Anergic B cells are hyporesponsive, short-lived, and chronic exposure to antigen in these cells is associated with decreased BCR expression [3]. The third type of tolerance mechanism, called **receptor editing**, leads to the internalization of the self-antigen-BCR complexes and to the activation of intracellular signals that initiate secondary light chain rearrangements [4]. Immature B cells migrate from the bone marrow to the periphery as **transitional 1 and 2** (T1 and T2) **B cells** (new emigrant B cells). T1 B cells precede T2 B cells which are considered the immediate precursors of naïve B cells. Both types of transitional B cells are short-lived, and only 10-30% of these cells enter the mature naïve B cell compartment [3, 4]. **Transitional 3** (T3) B cells, which were considered to be part of the linear development from immature to naïve B cells, are now believed to represent self-reactive anergic B cells [5]. Follicular mature, germinal center, and plasma B cells belong to the B-2 lineage. A second lineage of B cells called B-1 is found mainly in the peritoneum [6]. The mechanism regulating the transition between transitional B cells to mature naïve B cells is poorly understood, but it is known that this process mainly takes place in the spleen and the BCR signalling has an important role in this transition as well in the decision fate between the follicular and marginal zone pathway of B cell differentiation [3, 4]. Receptor editing may also occur in transitional B cells, but in less extent since these cells express low RAG [3].

In particular, the BCR signalling together with the B cell-activating factor receptor (BAFF-R or BR3) guides the selection of immature B cells after their exit from the bone

marrow [7]. BAFF is a fundamental survival factor for B cells which has shown to have an important role in the differentiation of T2, mature and MZ B cells [6].

Indeed, BAFF-/- mice are able to generate and release normally immature B cells from the bone marrow, but in the spleen these B cells stop at the T1 stage and fail to proceed to the T2 stage [8]. Moreover MZ B cells tend to disappear in the absence of BAFF, suggesting that BAFF is also required for their development [6]. Regarding the role of BAFF on the development of B-1 B cells, which is a unique population of mature cells found mainly in the peritoneum, there are data in mice deficient for BAFF showing a normal B-1 population, suggesting an alternative developmental pathway for these cells [6].

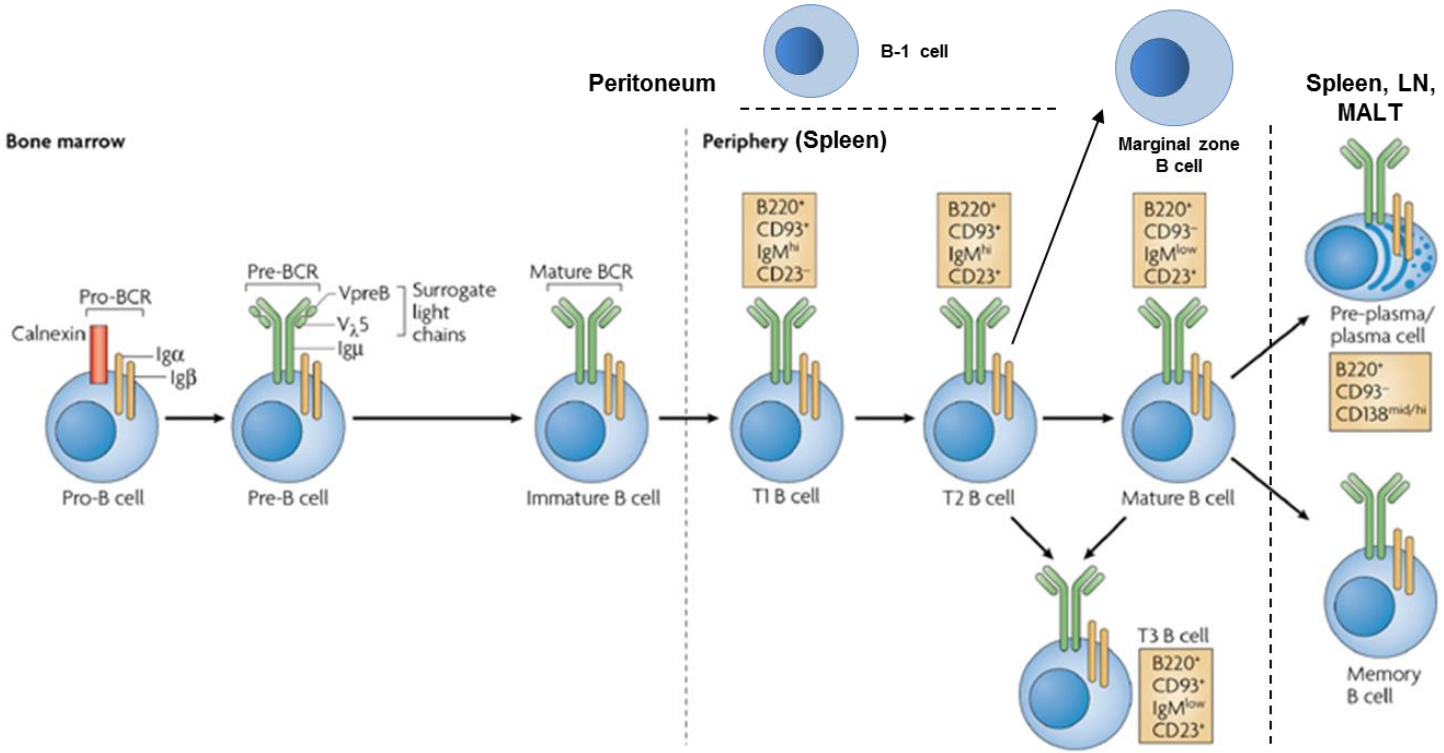

Figure 1.1 Stages of B cell development in both bone marrow and peripheral lymphoid tissue.

Events corresponding to each stage of B cell maturation from a pro-B cell to a mature B lymphocyte are illustrated.

Source: Cambier et al., Nature Reviews Immunology 2007

1.1.3 V(D)J recombination process.

The human Ig molecule consists of two identical heavy chains (IgH) and two identical light chains (IgL), each of which contains a variable domain for antigen recognition and a constant region for the effector functions. The Ig is encoded by three independent gene loci, with the IgH genes (for the heavy chain) located on chromosome 14, and the Igκ and Igλ genes (for the light chain) located on chromosome 2 and 22, respectively (**Figure 1.2**).

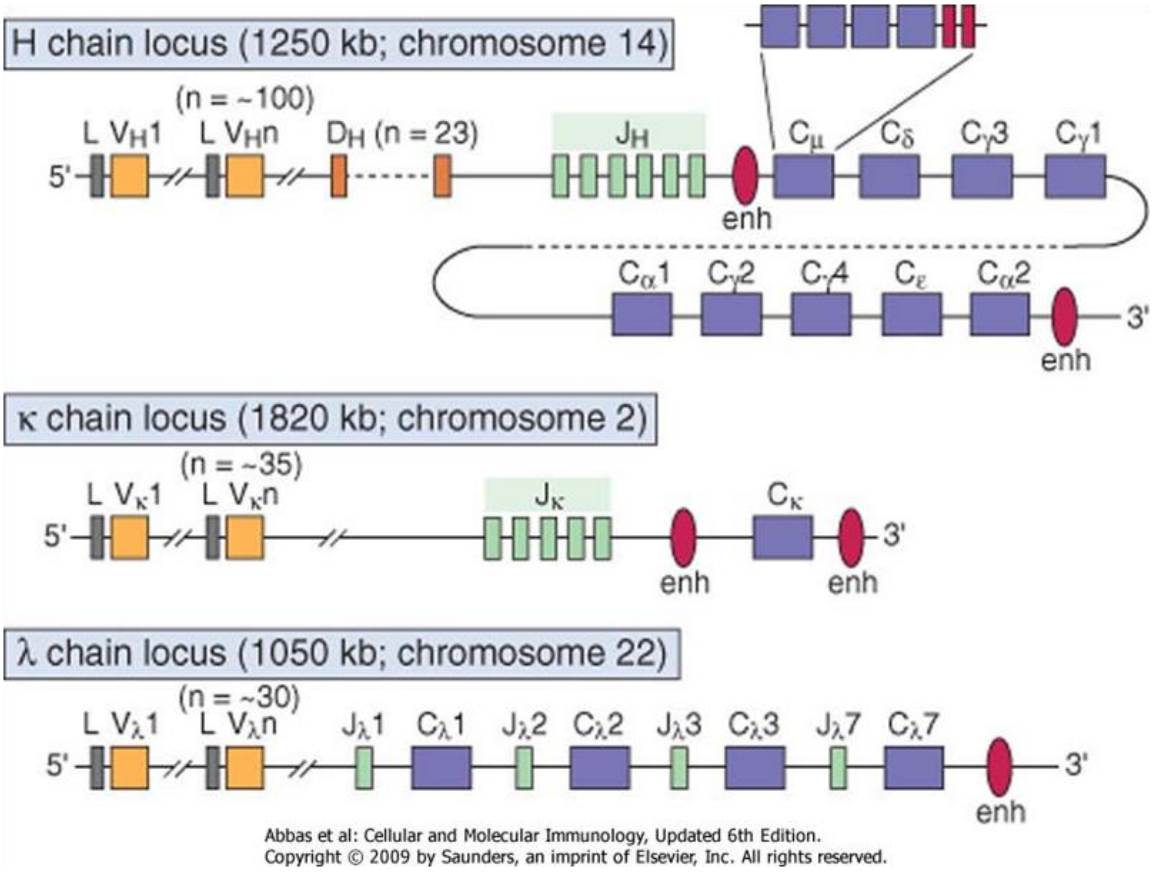

Figure 1.2 Human Ig loci organization.

Schematic diagram of the human heavy chain, κ light chain, and λ light chain loci. Only functional genes are shown. Variable (V), diversity (D) and joining (J) gene segments are represented in yellow, orange, and green rectangles, respectively. Constant (C) gene segments are shown in violet rectangles, enhancer elements as red ovals. Leader sequences (or signal sequences) are represented in grey.

The IgH locus consists of 123-129 VH gene segments of which 79 are pseudogenes and 39 are functional genes, 27 D gene segments, and 6 JH functional gene segments. The VH gene segments can be classified into seven different families (VH1-VH7) as well as the D gene segments [9, 10]. The Igκ locus consists of 76 Vκ genes belonging to 7 subgroups of which 31 to 36 are functional and 5 Jk gene segments [10]. Finally, the Igλ locus consists of 73 to 74 genes of which 14 are pseudogenes and 29 to 33 are functional, and 7 to 11 J gene segments [10]. Assembly of V-D-J gene segments by V(D)J recombination process is the central mechanism that leads to Ig antigen receptor diversity. This process is started by a protein complex consisting primarily of the Rag1 and Rag2 proteins (RAG complex). RAG binds and cleaves the DNA at specific sites called recombination signal sequences (RSSs) that flank each V, D and J gene segment [11]. The RSSs contain two conserved DNA elements – the heptamer and the nonamer, divided by a spacer of 12 (12RSS) or 23 (23RSS) base pairs. Normally, a 12RSS recombines only with a 23RSS, the so called 12-23 rule [11]. The ability of RAG to start V(D)J recombination relied on the accessibility of RSSs within the chromatin [11, 12]. As shown in **Figure 1.3**, V(D)J recombination starts when the RAG complex, probably together with high-mobility group protein B1 (HMGB1), binds to the 12RSS or the 23RSS, forming a signal complex [11]. Capture of the second RSS (synapsis) results in the formation of a paired complex. This step is followed by DNA cleavage by RAG (double strand breaks) between the gene segment and the RSSs, with the formation of hairpin loop at the end of the gene segment. In the second phase, the hairpin is opened and RAG cooperate with non-homologous end joining (NHEJ) factors and terminal deoxynucleotidyl transferase (TdT) for the nucleotides addition to rejoin the

DNA ends and form the coding joint [11]. Finally, RSSs ends are joined to form the signal joint.

1.1.3.1 Structural characteristic of the immunoglobulin antigen binding site.

Most sequence differences among different antibodies are confined in three short regions, named complementary determining regions (CDRs), which are embedded into four conserved framework regions. Altogether CDRs and FR regions form the variable region of the Ig. Starting from the amino terminus of both VH and VL, the CDRs are called CDR1, CDR2, and CDR3. The latter is the most variable region of the CDRs. The FR1, 2 and 3 together with the CDR1 and CDR2 are encoded by the V gene segments. The FR4 is encoded by the J gene segment. The VH-D-JH join forms the CDR3 which is the center of the antigen binding site. Although the antigen binding is mainly associated with the CDRs, the FR regions may also interact with the antigen.

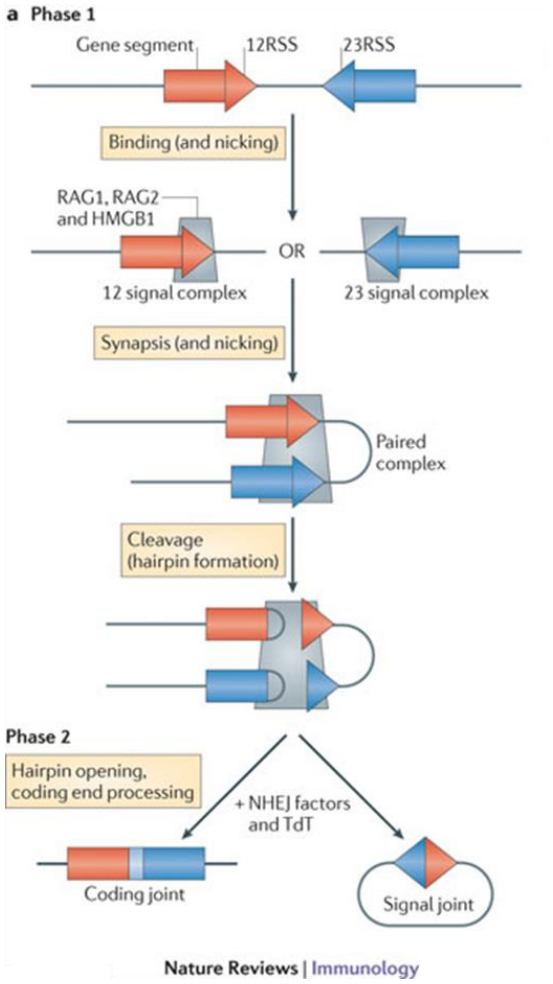

Figure 1.3 V(D)J recombination.

V(D)J recombination steps are shown. In the phase 1, the RAG complex binds the signal sequences forming a 12 or 23 signal complex. This step is followed by the formation of a paired complex with the subsequent cleavage by RAG proteins and hairpin loop formation. In the phase 2, the hairpin is opened and the gene segments are joined to form the coding joint.

Source: Schatz and Ji, Nature Reviews Immunology 2011

1.1.4 B cell differentiation after antigen encounter.

After naïve B cells bind protein antigens, they migrate into the outer T cell zones in secondary lymphoid organs (SLOs) where they receive cognate T cell help and start to differentiate following one of two pathways: i) follicular which gives rise to germinal centers (GCs), or ii) extrafollicular from which short-lived plasma cells (PCs) develop [4].

1.1.4.1 The germinal center (GC) reaction.

B cell affinity maturation takes place in structure called GCs, leading to the generation of memory B and plasma cells which produce high-affinity antibodies [13]. These structures were first described by Walther Flemming in 1884 as distinct regions of SLOs that contained dividing cells [14, 15]. GCs are the main site of somatic hypermutation (SHM) and affinity-based selection. They develop in the center of primary follicles composed of naïve B cells within SLOs such as spleen and lymph nodes (LN) after exposure to antigens, forming secondary follicles. Naïve B cells are pushed outside the developing GC, forming the B cell mantle zone. As shown in **Figure 1.4**, GCs can be divided in two distinct compartments, the dark and the light zone (DZ and LZ, respectively). The DZ, proximal to the T cell zone, is made of B cells with a high nucleus/cytoplasm ratio, thus appearing dark by light microscopy. The DZ is the site of B cell clonal expansion and antigen receptor diversification. The B cells in the DZ are called “centroblasts” due to their larger size and high-level of proliferation. The LZ, proximal to the LN capsule or to the spleen marginal zone, consists of B cells scattered among a network of follicular dendritic cells (FDCs), giving this zone a light appearance.

The LZ is the site of antigen selection and the B cells within this zone are referred to as “centrocytes” because they are smaller and more quiescent. Shuttling between the DZ and LZ is regulated by the expression of unique chemokine receptors, as described later in this thesis (Chapter 1.4). The LZ contains also naïve B cells, which are always in transit through the GC, T cells (mainly CD4+), as well as a small number of conventional dendritic cells. Tingible-body macrophages, a specialized group of phagocytes that clear dying B cells during GC selection, are also found throughout the GC [14].

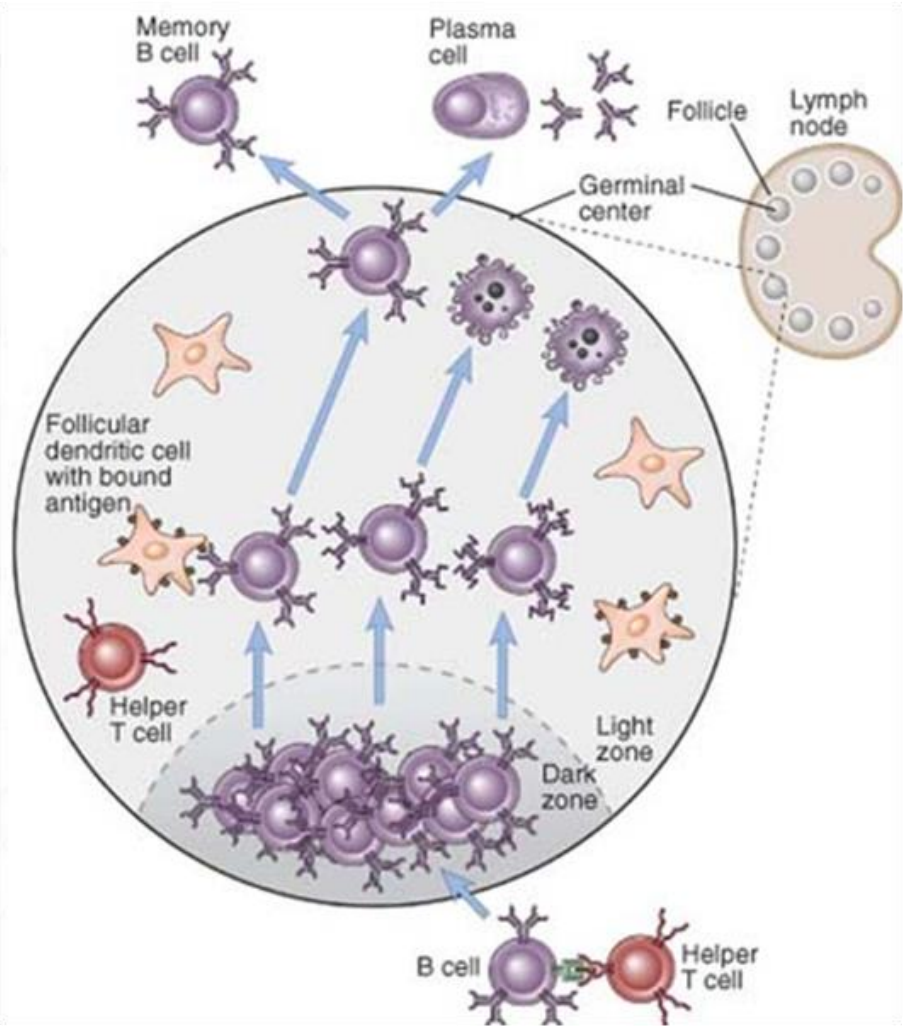

Figure 1.4 Schematic representation of a GC reaction in a lymph node.

GC B cells are the fastest dividing mammalian cells with a cell cycle between 6 and 12 hours [14, 16-19]. GC B cells express the enzyme activation-induced cytidine deaminase (AID), which is essential for SHM and class-switch recombination (CSR). SHM is a process characterised by the introduction of single point mutations in hot spots within the Ig variable genes which encode for the antigen-binding region of the antibody, resulting in an increased antibody affinity for antigens [20, 21]. Following SHM, B cells undergo CSR which affects the constant Ig region resulting in the production of different Ig isotypes (IgG, IgE, and IgA) responsible for the effector capacity of the antibody (**Figure 1.5A**) [22].

The transcriptional factor Bcl-6 is another important regulator of the GC B cell phenotype, and is upregulated during the GC reaction. This transcriptional factor has different functions. First, it silences the anti-apoptotic molecule Bcl-2 in GC B cells, a way to prevent autoimmunity that could arise as a result of SHM. Second, Bcl-6 contributes to the ability of GC B cells to tolerate DNA damage, as consequence of rapid proliferation and AID activity. Third, Bcl-6 silences the regulator of plasma cell differentiation Blimp-1 as well as key mediators associated with both the B cell receptor (BCR) and the CD40 signalling [14].

Bcl-6 expression is not only restricted to GC B cells but is expressed also by a subset of T cells, called T follicular helper cells (T_{FH} cells). T_{FH} cells are an important subset of effector T cells in the GC which provide a helper function to B cells. For long time the subset of T cells considered important in providing B cell help were the Th2 cells producing cytokines like IL-4 and inducing isotype switching to IgG and IgE [23]. Nowadays, it is apparent that T_{FH} cells are the real helper T cells to antigen-specific

naïve B cells. T_{FH} cells are different from Th1 and Th2 cells by several conditions, i.e., expression of the chemokine receptor CXCR5, localization in the B cell follicle, transcription factor (Bcl-6), and function [23]. Regarding their function, T_{FH} cells release IL-21 a helper cytokine which support B cell proliferation, isotype switching, and differentiation [23].

Another important component of the GC is the FDC network. FDCs retain unprocessed antigen on their surface in the form of immune complexes, and are believed to serve as an antigen reservoir during the GC reaction [14, 24, 25]. FDCs are also thought to support GCs by secreting chemokines and cytokines. Indeed, FDCs produce CXCL13 which is the major chemoattractant for B cells and follicular T cells. They also express cytokines such as IL-6 and BAFF (B cell-activating factor belonging to the TNF family), the latter being an important B cell survival factor that promotes the survival and differentiation of B cells *in vitro* and *in vivo* [14].

1.1.4.2 Activation induced cytidine deaminase (AID).

As reported above, AID is essential for SHM of the antibody variable regions and CSR of the constant regions. AID belongs to a family of cytidine deaminases sharing sequence homology with the RNA editing cytidine deaminase APOBEC1 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1) (**Figure 1.5B, top panel**) [26]. Both SHM and CSR are started by AID by converting deoxycytidine (dC) to deoxyuracil (dU) [26]. The U:G mismatches can be then processed and removed by several mechanisms (**Figure 1.5B, bottom panel**). Normally, the mechanisms used to repair the mismatches are the base excision repair (BER) and the mismatch repair (MMR) pathways. The first one is

considered the main pathway during which the deoxyuridine in the switch (S) region DNA introduced by AID, is removed by the uracil DNA glycosylase (UNG) enzyme that generates an abasic site processed by the apurinic/apyrimidinic endonuclease 1 (APE1). The nicks generated are finally processed creating a blunt double strand break (DSB) that can then be ligated to other breaks on downstream S region DNA to complete CSR process [27]. The MMR machinery includes the mutS homologue 1 (MSH1), MSH6, exonuclease 1 (EXO1), mutL homologue 1 (MLH1) and post-mitotic segregation (PMS) proteins which introduce nicks on S region DNA, that are then processed similarly to the BER pathway [27]. AID targets the DNA not randomly but on specific hotspots defined by the sequences DGYW or WRC (D=A, G or T; Y=C or T; W=A or T; R=A or G) and they reverse complements [28]. AID expression is normally restricted to GC B cells undergoing CSR and SHM but also to a new subset of B cells, called interfollicular (IF) large B cells which will be described in Chapter 1.4.

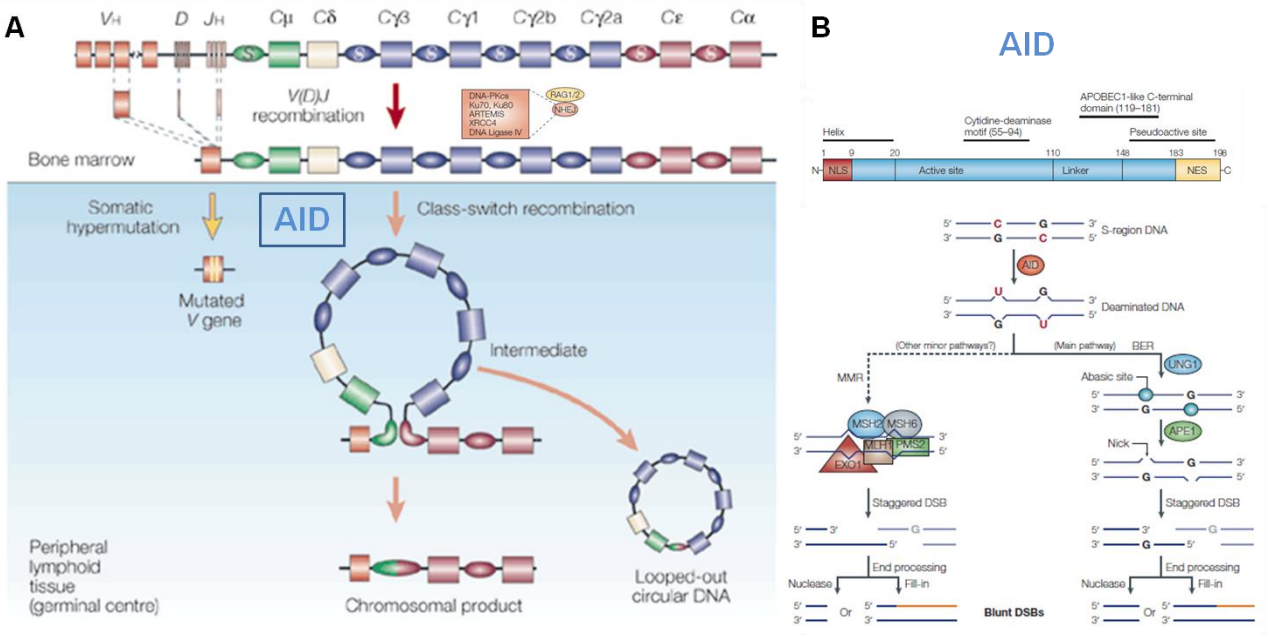

Figure 1.5 Rearrangement of the Ig heavy chain locus.

(A) Schematic representation of V(D)J recombination which assembles the variable (V), diversity (D) and joining (J) gene segments in the bone marrow. In the periphery somatic hypermutation (SHM) and class-switch recombination (CSR) occur and they are both dependent on the B cell specific enzyme activation induced cytidine deaminase (AID). CSR exchanges the Cμ constant region for downstream constant regions, such as Cγ, Cα or Cε. **(B)** Top: AID structure is shown. It consists of a nuclear-localization sequence (NLS), a nuclear-export sequence (NES), and other domains based on the structure of APOBEC1 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1) with whom AID shares sequence homology. Bottom: the mechanisms used to repair the U-G mismatch introduced by AID are represented (on the right, base excision repair pathway (BER) and on the left, mismatch repair (MMR) pathway). Processing the mismatches can lead to double stranded breaks (DSBs) formation and then to class-switch recombination.

Source: adapted from Chaudhuri and Alt, Nature Reviews Immunology and Kinoshita and Honjo, Nature Reviews Molecular Cell Biology 2

1.1.4.3 Extrafollicular antibody production.

Extrafollicular antibody responses are considered the quickest way to produce antibodies after antigen encounter. It can be divided into several phases. After antigen binding, B cells migrate to the T cell rich area of the SLOs (primarily the spleen or Peyer's patches in the gut) where they interact with antigen-primed T cells. This interaction induces local growth of both cell subsets. After two cell cycles, the B blasts which were induced to become plasmablasts move to the local extrafollicular site where they proliferate and eventually differentiate into short-lived plasma cells without contact with T cells [29]. Extrafollicular foci can last only for few days, whereas GCs persist for weeks. The signals that lead the B cells to GC formation or to extrafollicular growth as plasmablasts are not well defined. It has been proposed that certain antigens may preferentially recruit one subset of B cells (i.e. marginal-zone like B cells) to extrafollicular growth and another subset (i.e. follicular B cells) to follicular differentiation but without absolute restriction to a single B cell subset [29]. Additionally, it has been suggested that cells with high affinity BCR move to extrafollicular sites where they differentiate into short-lived plasma cells, whereas low affinity B cells are more common in the GC environment that gives rise to long-lived plasma cells and quiescent memory B cells, thus providing long-term immunological protection [30, 31].

1.1.5 Self-tolerance checkpoints during B cell development.

In 1901 Paul Ehrlich was the first to speculate that an individual is able to produce toxic autoantibodies. He suggested that the immune system can discriminate between self-

and non self-antigens thus preventing the so called “horror autotoxicus” [32]. To avoid the development of autoimmune diseases, the immune system has introduced several mechanisms to discriminate between potentially dangerous autoantibodies and protective antibodies [31]. As described above, during B cell development the main mechanisms that remove or silence self-reactive antibodies are mainly three: receptor editing, deletion and anergy. The maintenance of self-tolerance in the human antibody system is of critical importance since a large number of autoimmune diseases (i.e., systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren’s syndrome (SS), multiple sclerosis, myasthenia gravis, Graves’ disease, and others) are characterised by high titers of autoantibodies in the patients’ sera, reflecting a failure in the self-tolerance mechanisms that control the production of autoreactive B cells, thus underlying abnormalities in B cell self-tolerance checkpoints [31].

In order to characterise the number of human B cells that produces autoantibodies at each step of differentiation and thus to understand how the generation of autoreactive B cells is regulated, Michel Nussenzweig with his fellows Hedda Wardemann and Eric Meffre developed an approach to clone and express recombinant antibodies from single B cells at different stages of their development (see paragraph 1.1.5 and Chapter 2) [33, 34]. They found that a large percentage (55%) of newly generated early immature B cells in the human bone marrow are polyreactive towards different self- and non-self antigens such as DNA, lipopolysaccharides (LPS), and insulin while up to 75% are self-reactive against nuclear antigens (ANA reactivity) (**Figure 1.6**) [33]. These percentages strongly decrease during the transition from early immature B cells to immature B cells (55 to 7% for polyreactivity and 75 to 40% for ANA reactivity,

first self-tolerance checkpoint). It has been proposed that Ig receptor editing has the major role during this first checkpoint in the bone marrow [31, 33, 35]. Indeed, *in vitro* studies in humans have revealed that autoreactive antibodies in the early immature B cell compartment can go through receptor editing with Ig light chain replacement to regain a normal phenotype [31, 36-38]. Moreover, the same group discovered a second self-tolerance checkpoint during the differentiation from transitional to mature naïve B cells. During this transition the number of ANA reactive B cells decreases significantly [33]. However, 20% of mature naïve B cells still display low levels of self-reactivity and 6% are polyreactive [33]. This physiological small percentage of polyreactive and autoreactive circulating naïve B cells is believed to form a reservoir of "natural" antibodies, to contribute to peripheral T cell tolerance and to play an important role in the immediate response to invading pathogens [39]. A third self-tolerance checkpoint ensures the removal of most poly- and self-reactive antibodies during the IgM+ memory B cell differentiation (pre-GC checkpoint). However, B cells can acquire again poly- and self-reactivity during the GC reaction as consequence of somatic hypermutation. As a result, polyreactivity increases to 20% and self-reactivity to 40% in the IgG+ memory B cell subset [40]. Finally, a fourth tolerance checkpoint has been suggested in order to ensure that only a small fraction of the memory B cell antibodies is polyreactive and non-pathogenic [39].

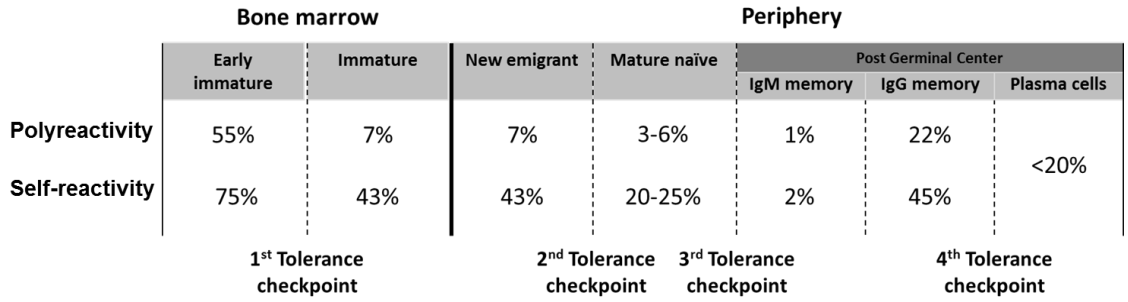

Figure 1.6 Tolerance checkpoints during human B cell development.

Newly generated early immature B cells in the bone marrow of healthy donors express polyreactive (55%) and self-reactive antibodies (75%). Highly polyreactive and self-reactive antibodies are efficiently removed at the first tolerance checkpoint, leading to a significant reduction in polyreactivity (7%) in immature B cells in the bone marrow. However, a significant percentage of immature B cells retain low levels of self-reactivity (43%). Self-reactive immature B cells leave the bone marrow as new-emigrant B cells moving to the peripheral blood. In the periphery, counterselection of self-reactive new emigrant B cells occurs again at the second tolerance checkpoint before maturation into naïve B cells (~6% polyreactivity and ~20% self-reactivity). Polyreactive and self-reactive B cells are efficiently removed from the circulating IgM+ memory B cell compartment at a third tolerance checkpoint. However, polyreactivity and self-reactivity increase again in circulating IgG+ memory B cells (45% and 22%, respectively), the majority of which acquires self-reactivity as a consequence of somatic mutation. The last tolerance checkpoint counterselects the entrance of polyreactive and self-reactive memory B cells into the long lived plasma cell compartment [33, 39, 40].

1.1.5.1 Disturbances of self-tolerance checkpoints during autoimmune diseases.

Several groups have demonstrated that tolerance checkpoints are defective in patients with autoimmune diseases, i.e. SLE [41, 42], RA [43] , and type 1 diabetes [44]. The loss of self-tolerance that leads to the accumulation of autoreactive and polyreactive antibodies is associated with defects in the first and/or second tolerance checkpoint. Abnormalities in the first tolerance checkpoint are reflected by abnormalities in the immature B cell compartment in the bone marrow [31]. Since the antibody repertoire of new emigrant B cells is similar to that of immature B cells [33], defects in the first checkpoint are normally associated with abnormalities in the new emigrant B cell subset. Conversely, increased level of poly- and self-reactivity in the mature naïve B cell compartment reveals abnormalities in the second self-tolerance checkpoint [31]. Using the single cell cloning strategy to generate recombinant antibodies from B cells isolated from SLE patients, the Nussenzweig laboratory found an increased number of self-reactive and polyreactive antibodies in both the new emigrant and mature naïve B cell compartments [42]. Similar results were obtained by the Meffre laboratory in RA patients using the same technique. They showed that autoreactive B cells increase significantly between 30 and 50% in the mature naïve B cell compartment of RA patients compared to normal individuals (20%, see **Figure 1.6**) [43]. This abnormal accumulation is most likely the result of early defects in central self-tolerance checkpoint in the bone marrow, as demonstrated by increased autoreactivity of new bone marrow emigrant B cells, also associated with peripheral defects in reducing autoreactive B cells from the mature naïve recirculating B cell pool [43]. Despite the

evidence that the naïve B cell compartment is enriched with poly- and self-reactive antibodies in patients with different autoimmune diseases, whether similar defects in the B cell self-tolerance checkpoints in early stages of the B cell development are also associated with SS is currently unknown.

1.2 Humoral autoimmunity in Sjögren's syndrome.

1.2.1 Introduction.

The first description of Sjögren's syndrome (SS) is generally associated with Mikulicz who in 1892 described a case study of a patient with enlargement of parotid and lacrimal glands characterised by a small-round-cell infiltrate [45]. However, the first report of the disease, today known as SS, was given in 1932 by Henrick Sjögren, a Swedish ophthalmologist who described a triad of keratoconjunctivitis sicca, xerostomia and rheumatoid arthritis (RA).

SS is a chronic autoimmune disease characterised by immune cell infiltration of the exocrine glands leading to glandular dysfunction with clinical symptoms of xerostomia (dry mouth) and keratoconjuctivitis (dry eyes), autoantibodies production and extraglandular systemic manifestations, including fatigue, arthritis and diverse organs involvement [46]. Moreover, it has been estimated that around 5% of patients with SS develop extranodal B cell lymphomas [47]. SS can be primary (pSS), or it can occur secondary to various other autoimmune diseases, such as RA, scleroderma, or systemic lupus erythematosus (SLE).

1.2.2 Sjögren's syndrome classification criteria.

Several classification criteria of SS have been proposed in the last 30 years. The most accepted and better validated criteria for SS are those included in the revised version of 2002 of the European criteria proposed by the American-European Consensus Group [48]. The American-European criteria consist of six elements; each of them is

divided into one to three criteria (**Table 1.1**). The criteria combine symptoms of dry eyes and dry mouth, ocular signs, e.g., Schirmer's test positivity which measures the reduction of tear flow, histopathology characterisation with positive minor salivary glands biopsy results (according to Chisholm and Mason focus score [49]), and the presence of anti-Ro/SSA and/or anti-La/SSB autoantibodies [48]. Diagnosis of primary SS requires four out of six of these criteria. One of these four criteria must include a positive salivary gland biopsy or the presence of autoantibodies [50].

Revised international classification criteria for Sjögren's syndrome
I. Ocular symptoms (at least one positive response to one of the following question)
1. Have you had daily, persistent, troublesome dry eyes for more than three months?
2. Do you have a recurrent sensation of sand or gravel in the eyes?
3. Do you use tear substitutes more than 3 times a day?
II. Oral symptoms (at least one positive response to one of the following question)
1. Have you had a daily feeling of dry mouth for more than three months?
2. Have you had recurrently or persistently swollen salivary glands as an adult?
3. Do you frequently drink liquids to aid in swallowing dry food?
III. Ocular sign - at least one of the following two tests:
1. Schirmer's test, performed without anaesthesia (≤ 5 mm in 5 minutes)
2. Rose bengal score or other ocular dye score
IV. Histopathology. In minor salivary glands focal lymphocytic sialoadenitis with a focus score ≥ 1, defined as a number of lymphocytic foci per 4 mm^2 of glandular tissue
V. Salivary gland involvement. Objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests:
1. Unstimulated whole salivary flow (≤ 1.5 ml in 15 minutes)
2. Parotid sialography
3. Salivary scintigraphy
VI. Autoantibodies: presence in the serum of the following autoantibodies:
1. Antibodies to Ro(SSA) or La(SSB) antigens, or both
Exclusion criteria
Past head and neck radiation treatment
Hepatitis C infection
Acquired immunodeficiency disease (AIDS)
Pre-existing lymphoma
Sarcoidosis
Graft versus host disease
Use of anticholinergic drugs (since a time shorter than 4-fold the half life of the drug)

Table 1.1 Classification criteria for Sjögren's syndrome [48].

1.2.3 Aetiology.

The aetiology of SS is unknown. However, similarly to the majority of autoimmune disorders, it is likely that a multifactorial combination of genetic background, gender, immunological alterations and environmental factors is implicated in the disease development.

1.2.3.1 Genetic background.

A strong association to specific MHC alleles has been shown in SS, in particular HLA-DR3, HLA-B8 and HLA-Dw3 has been observed in patients affected by primary SS compared with normal individuals [51, 52]. In addition, an association between the haplotype DR3-DQA1*0501-DQB1*02 and the production of anti-Ro/La antibodies in patients affected by SS and SLE has been reported, suggesting a partial overlap in the genetic background of these diseases and in the immunological abnormalities leading to the production of the Ro/La autoantibody specificities [53]. More recently, associations of gene polymorphisms outside the HLA-locus in SS have been characterised. In particular, association of IRF5 and STAT4 gene variants have been found in a cohort of SS patients; both genes are components of the interferon type-I (IFN-I) pathway [52].

1.2.3.2 Environmental factors.

Infection agents have been particularly linked to SS development. Glandular viral infection could induce activation of HLA-independent innate immune system through Toll-like receptors, leading to inflammatory cytokines production [54].

Cytomegalovirus, Hepatitis C virus (HCV, currently among the exclusion criteria for SS), Human Immunodeficiency virus (HIV) and Human T Cell Leukemia Virus type 1 (HTLV-1) have all been associated with the development of a chronic sialadenitis that mimics SS and the occurrence of a SS-like syndrome in humans and in mouse models [52]. Epstein-Barr virus (EBV) has been shown to be present as a latent infection in human salivary glands [55] and, upon reactivation, EBV seems able to induce T cell-dependent B cell activation together with cytokine production [46, 56].

1.2.3.3 Gender.

SS is characterised by a strong female prevalence - the female:male ratio is 9:1, suggesting the presence of sex-specific predisposing factors. It most commonly affects women aged 40-60. Lack of estrogens has been associated with predisposition to develop SS. This data has been supported by the fact that in the majority of patients the disease is expressed in the perimenopausal period of life of women [52]. Moreover, studies in normal mice and in murine experimental models showed that estrogens can suppress the development of SS, enhance T cell recruitment in salivary glands and prevent cell death in the lacrimal glands [52, 57, 58].

1.2.4 Extraglandular systemic manifestations.

Besides exocrine glands, SS can affect several other organs including joint, skin, gut, respiratory tract, and neurological system. A briefly overview of the main conditions associated with SS will be reported in the following section.

- Joint involvement in SS is common, especially among women and it affects mainly small joints, symmetrically associated with mild synovitis. The development of a proper erosive arthritis has been rarely reported [59].
- The skin is not infrequently involved in patients with SS. Raynaud's phenomenon can occur in one third of SS patients [60]. Less common skin manifestations include vasculitis which can affect small and medium sized vessels, urticaria vasculitis and annular vasculitis.
- The most common gastrointestinal manifestation is dysphagia, i.e. difficulty in swallowing [61]. Association with primary biliary cirrhosis as well as coeliac disease has been also observed in patients with SS [62]. Signs of sicca syndrome, presence of anti-nuclear antibodies (ANA), mixed cryoglobulinemia, anti-mitochondrial antibodies, anti-Ro/SSA, rheumatoid factor and development of salivary gland infiltrates have been correlated with liver dysfunction during HCV infection [63, 64].
- Respiratory tract manifestations of the upper airways have been widely correlated with the decreased function of the glands of the nose, trachea and bronchi [65]. Lower respiratory tract involvements are associated with lymphocytic interstitial pneumonitis, rarely with alveolitis and mild obstructive or restrictive pulmonary deficit [66].
- Neurological manifestations can involve both the central and more commonly the peripheral nervous system, including peripheral neuropathy, mononeuropathy and autonomic neuropathy [45].
- There is an increased risk of congenital heart block in babies with mothers positive for anti-Ro/SSA autoantibodies

- Autoimmune hypothyroidism is commonly associated SS [45].
- Patients with SS have an increased risk of developing lymphoma (up to 44 fold). Usually the lymphoma arises within the major salivary glands (e.g., parotids) and most often is a low-grade marginal zone B cell lymphoma of the mucosa-associated lymphoid tissue (MALT-L) [67].

1.2.5 Immunopathogenesis of salivary gland inflammation.

Salivary gland inflammation during SS is sustained by the upregulation of adhesion molecules and the production of several chemokines and cytokines which lead to the migration into the gland of immune cells [54]. Beside the immune cell component, epithelial cells in SS have also an active role in the initiation and perpetuation of inflammation and the disease has also been described as an "autoimmune epithelitis". The upregulation of adhesion molecules and the production of chemokines, cytokines, and B cell survival factors such as BAFF by the epithelial cells is related to their potential role in promoting an aberrant migration, survival and proliferation of DCs, T cells and B cells in the inflamed SG [54]. T cells and DCs in the salivary gland contribute to the production of inflammatory cytokines (e.g., IL-1β, IFN-γ, TNF) and thus to enhance the dysregulation and induction of epithelial cell apoptosis. Through apoptosis the epithelial cells present intracellular autoantigens, such as Ro/SSA and La/SSB which promote the production of autoantibodies by the B cell infiltrates [54].

1.2.5.1 Organization of the inflammatory infiltrates.

Although SS is defined as systemic autoimmune disease, it can be included within the organ-specific autoimmune disorders since it affects mainly exocrine glands. In

particular, minor salivary glands have been selected as histological readout for SS since they are commonly involved in the disease course and for the easy accessibility to their tissue during surgical procedures. Salivary glands (**Figure 1.8**) can be divided in major (parotid, submandibular and sublingual) and minor glands (labial, buccal and palatine). The acini represent the secretory component within the glands.

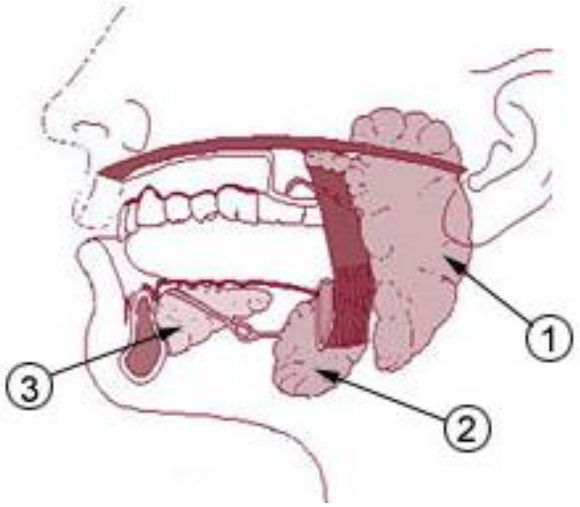

Figure 1.7 Salivary gland illustration.

(1) Parotid, (2) submandibular gland and (3) sublingual gland.

In particular, in SS glands, focal infiltrates of T and B cells, DCs and macrophages organize characteristically around ductal epithelial cells, particularly of striated and intercalated ducts. The definition of focal infiltrates follows the classification originally described by Chisholm and Mason, and refers to a periductal aggregate of at least 50 mononuclear cell/4mm^2 [49, 68]. Hence, T and B lymphocytes play a central role in the immunopathogenesis of this disease, though which set of lymphocytes has the principal role is still debated [69]. It has been proposed that T cells may play the principal role in the early immunopathogenesis of SS while there are strong evidences to suggest that B cells play the leading role in this autoimmune disease at later stages of chronicity, i.e. by promoting a local immune responses to allo- and auto- antigens by antibody-dependent and antibody-independent mechanisms [69, 70]. Indeed, around 20-30% of the infiltrating cellular component within the gland is constituted by B cells [71] and normally, during the expansion and organization of the periductal inflammatory focus, a progressive increase in the number of recruited B cells can be observed. In a number of SS patients, B and T lymphocytes can become highly organized, with T/B compartmentalization, and in around 30-40% of SS minor (labial) salivary glands the presence of ectopic germinal centers (GC)-like structures characterised by CD21+ follicular dendritic cells (FDCs) networks formation and peripheral node addressin (PNAd) expression has been demonstrated (**Figure 1.7**) [72, 73]. This phenomenon is known as ectopic lymphoid neogenesis and has been described in several chronic inflammatory conditions and often related to the development of autoantibodies and to a more severe disease outcome [74]. In several chronic inflammatory conditions the formation and maintenance of these ectopic

lymphoid structures (ELS) is critically dependent on the ectopic expression of lymphotoxins (LT) and lymphoid chemokines CXCL13, CCL19, CCL21 and CXCL12 (see Chapter 1.4).

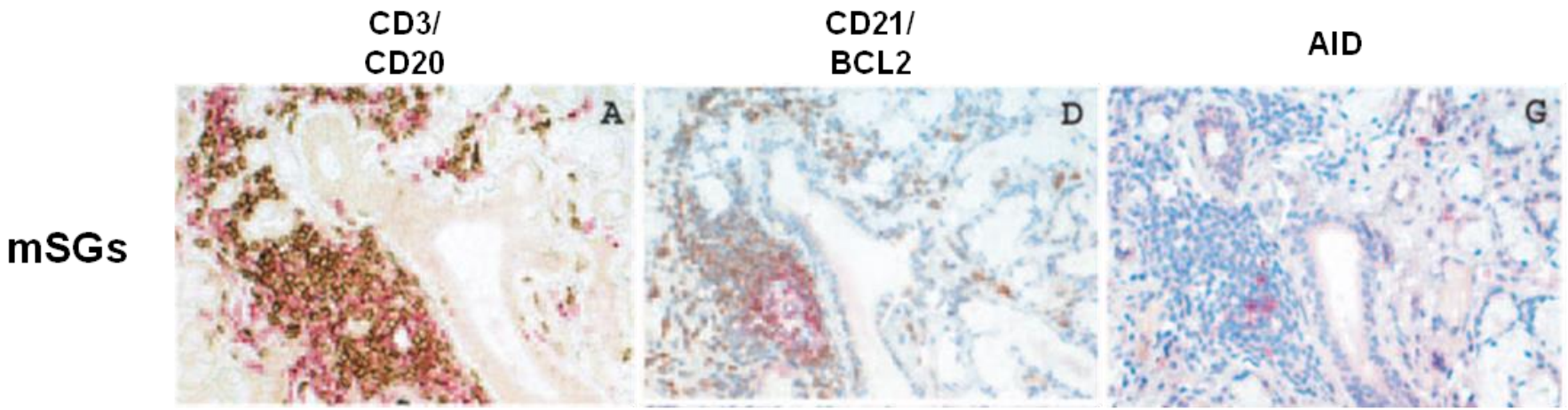

Figure 1.8 Ectopic GC-like structures in minor salivary glands (mSGs) of SS patients.

Representative example of ectopic GC-like structure in SS mSG is shown. Ectopic GCs are characterized by the classical T/B cell segregation, CD21+ FDC network formation and AID expression within the FDC network. Left panel: staining for CD3 (brown) and CD20 (purple); middle panel: staining for CD21 (purple) and Bcl-2 (brown); right panel: single staining for AID (purple).

Source: Bombardieri et al., The Journal of Immunology 2007

It has also been shown that ELS in SS are functional in promoting Ag-driven B cell activation, selection and proliferation with the in situ production of autoantibodies [73].

Physiologically, antigen-driven BCR selection via affinity maturation takes place within GC of SLOs where the processes of SHM and CSR of the Ig genes occur, as explained in Chapter 1.1. As previously reported, both CSR and SHM are initiated by and critically dependent on the expression of AID [75]. The important demonstration that AID is expressed in ELS both in minor SGs and parotids of SS patients has provided evidence that these structure are functional and provide the microstructural, molecular and cellular environment to sustain Ag-driven B cell activation and autoantibody production [73].

1.2.5.2 Peripheral B cell disturbances in patients with Sjögren's syndrome.

A significant advance on our understanding of B cell dysregulation in autoimmune diseases such as SS and RA, has been recently provided by studies focussing on quantitative and qualitative analysis of B cells subpopulations (i.e., frequency of autoreactive B cells) and peripheral B cell abnormalities. In humans, circulating B cell subsets are divided into three main groups based on the levels of CD27 expression [76, 77]. CD27 is a tumor necrosis factor receptor expressed on B and also T cells. It binds to ligand CD70 which is expressed on T cells (mainly activated CD4+ T cells) and activated B cells. The CD27/CD70 interaction is involved in the differentiation of B cells into plasma cells [78].

The three groups can be divided as follow:

1) CD27- B cells (~70% of circulating B cells) which include resting naïve Bm1 (IgM+IgD+CD23-), activated naïve Bm2 (IgM+IgD+CD23+) and pre-germinal center (IgD+CD38++CD10+) B cells;

2) CD27+ B cells (~30% of circulating B cells) which comprise early memory eBm5 (IgM+IgD+CD38+), memory Bm5 (IgM+IgD-CD38-CD77-CD44+), memory unswitched (IgD+CD38-) and switched (IgD-CD38-) B cells and plasmablast (CD138-).

3) The $CD27^{high}$ group (~1% of circulating B cells) is formed by early plasma cells (CD138+).

The recognition of different B cell subpopulations has led to the discovery that several autoimmune diseases display altered frequency of B cells subsets in the peripheral blood [76, 79-81]. In particular, patients with pSS are characterised by typical B cell disturbances with a predominance of circulating CD27- naïve B cells and a dramatic reduction of the percentage and total number of CD27+ memory B cells in the peripheral blood compared to healthy individuals [82]. The decrease in circulating memory B cells has been explained as a result of accumulation in the affected salivary glands [82], where lesional memory B cells have been shown to be characterised by heavily mutated VH genes and evidence of ongoing clonal diversification, strongly suggesting a local antigen-driven process [72]. The distribution of peripheral B cell subpopulations in patients with secondary SS is determined mainly by the associated rheumatic disorders [47].

1.2.5.3 Autoantibodies in Sjögren's syndrome.

SS is characterised by both organ specific and non-specific autoantibodies. Historically, anti-Ro/SSA and anti-La/SSB have been considered the two major autoantibodies associated with humoral autoimmunity in patients with SS but are also commonly detected in sera of patients with SLE. Other autoantibodies found in the sera of patients with SS include anti-nuclear antibodies (ANA), rheumatoid factor (RF), type II cryoglobulins (displaying monoclonal RF) and less frequently anti-mitochondrial antibodies (AMA), anti-centromere antibodies, and anti-smooth muscle antibodies [83]. In addition, other autoantibodies, including those against alpha-fodrin, carbonic anhydrase II and the muscarinic acetylcholine receptor 3 (M3R), have been described in patients with SS and suggested to exert a pathogenic role in this disease (i.e., inducing salivary dysfunction), especially the latter [84-87].

1.2.5.4 Anti-Ro/SSA and anti-La/SSB autoantibodies.

Anti-Ro/SSA and anti-La/SSB antibodies are directed against a complex of proteins (Ro 52 kDa, Ro 60 kDa and La 48 KDa) and small uridine-rich hY RNAs which are short (85-112 nt) RNA polymerase III transcripts [83, 88]. The protein component of both antigens is responsible for the immunogenicity since the autoantibodies are unable to recognise the isolated RNA molecules.

The protein part of the Ro antigen is mainly represented by the 60 KDa (528 amino acids) and 52 KDa (475 amino acids) proteins. Ro60 seems to be involved in the post-transcriptional regulation of 5S ribosomal RNA and other RNA polymerase III transcripts [83, 89]. Ro52 is a E3 ligase involved in ubiquitination [83, 90]. La is a

phosphoprotein (408 amino acids) with the capacity to bind the 3' uridine part common in all nascent RNA polymerase III transcripts, as well as the EBV RNAs EBER I and II and adenoviral VA RNAs [83, 88]. Its function is also associated with the regulation of RNA polymerase III transcription [83].

Anti-Ro/SSA and anti-La/SSB antibodies are found in around 60% and 40% of patients with SS, respectively. Normally, anti-Ro/SSA antibodies are detected either alone or in combination with anti-La/SSB, while anti-La/SSB are rarely found without anti-Ro/SSA [83, 88]. As mentioned above, both autoantibodies are part of the current classification criteria for SS and they are normally used as first screening to support the diagnosis of SS in patients suspected to suffer from this disease. Several techniques are used to detect anti-Ro/SSA and anti-La/SSB autoantibodies in clinical diagnostic. Normally, before anti-Ro/La determination, sera are screened by indirect immunofluorescence (IIF) assay on Hep-2 cells. Although both antibodies normally show a speckled nuclear pattern by IIF, there are evidence that anti-Ro antibodies can react with a fibrous cytoplasmic network similar to cytokeratin [91]. Thus, the presence of both autoantibodies is preferentially assessed using other methods, such as enzyme-linked immunosorbent assay (ELISA) which has been estimated to have a sensitivity of 72% and a specificity of 95%, Western blotting and immunodiffusion which are less routinely used due to the lower sensitivity and more complex procedure [83].

Finally, the increased risk to develop neonatal lupus and/or congenital heart block in a small minority of pregnant women positive for Ro antibodies is particular important among the several clinical manifestations associated with these autoantibodies, i.e.,

palpable purpura, lymphopenia, hypergammaglobulinemia and minor salivary gland infiltration [92, 93]. It has been proposed that maternal anti-Ro antibodies could cross the placenta and in this way damage the fetal cardiac conduction system [83].

1.2.5.5 Anti-muscarinic acetylcholine receptor 3 (M3R) autoantibodies.

To date no specific pathological autoantibodies have been identified in patients with SS. However, recent studies have proposed a possible role of autoantibodies directed against a muscarinic receptor in inducing glandular dysfunction in SS patients. Identification of pathogenic autoantibodies would be an important step in the management of SS for three main reasons: (i) their identification could form the basis for a diagnostic test, (ii) removal of the pathogenic antibodies could constitute a therapy for the patients, and (iii) better knowledge about the pathogenesis of SS could lead to the development of new therapies [84].

Muscarinic receptors (MR) are G-proteins coupled acetylcholine receptors [83]. So far, five subtypes of MR (M1R-M5R) have been identified; in particular, M3R seems to play important roles in endocrine and exocrine gland secretion (i.e., activation of salivary and lachrymal glands), smooth muscle contraction, and calcium concentration in the vascular endothelium [83, 94]. M3R expressed on salivary gland cells is activated by acetylcholine binding, inducing an increase in Ca^{2+} via inositol 1, 4, 5-trisphosphate (IP3) and IP3 receptor. This event activates the apical membrane Cl^- channels to induce saliva secretion. M3R activation also induces the aquaporin 5 (AQP5) water channel to move from the cytosol to the apical membrane, causing a rapid transport of water

across the cell membrane [87]. M3R is characterised by four extracellular domains. Recently, it has been suggested that among all these domains, the second extracellular loop may be responsible for the immunogenicity, meaning that anti-M3R autoantibodies against the second extracellular loop could contribute to the salivary dysfunction in patients with SS [87].

1.2.6 Treatment.

Untreated dry mouth and dry eyes symptoms in patients with SS can lead to oral diseases (i.e., dental loss, oral candidiasis, and periodontal disease) and corneal ulcers /perforation, respectively [95]. When other organs are affected, a delay in the therapy can potentially result, although rarely, in organ failure and death such as in the case of vasculitic lesions or active glomerulonephritis. Moreover, patients with SS have a higher risk to develop lymphoma than healthy individuals, as previously highlighted, a factor directly influencing morbidity and mortality in SS.

Dry eyes symptoms are normally treated with preservative-free teardrops, whereas for dry mouth mechanical or gustatory stimulations are used first [95]. However, the production of saliva can be increased by drugs such as pilocarpine and cevimeline which are muscarinic agonists.

Systemic therapy is directed by the extent of the extraglandular involvement and more in general disease severity. For instance, if a patient develops articular involvement, non-steroidal anti-inflammatory drugs (NSAIDs) can be used to provide relief from minor symptoms while methotrexate may be prescribed in the presence of inflammatory arthritis. To treat chronic symptoms, hydroxychloroquine can be used to

ameliorate fatigue, arthralgias, and myalgias symptoms. Finally, B cell targeted therapies (rituximab and belimumab) have shown to be more promising therapies compared to TNF blockers which are not efficacious in patients with SS. However, such B cell modulating agents are not routinely available for the treatment of SS in the NHS setting.

1.3 Humoral autoimmunity in Rheumatoid Arthritis.

1.3.1 Introduction.

Rheumatoid arthritis (RA) is the most common inflammatory erosive polyarthritis, affecting around 1% of the worldwide population. RA is associated with progressive disability, systemic complication - including cardiovascular, pulmonary, psychological, and skeletal disorders, early death, and socioeconomic costs [96, 97]. In RA, the lining of the joints - the synovium turns to be the site of an ongoing heterogeneous infiltration of leukocytes, synovial lining hyperplasia, and neovascularisation, which leads to cartilage destruction, bone erosion and, eventually, joint disability. The synovium is a loose connective tissue which is found between the joint capsule and the joint cavity of arthrodial joints (**Figure 1.9**). The normal synovium is composed mainly by two distinct layers: the intimal lining layer and the synovial sublining layer. The intimal lining layer is mainly composed by fibroblast-like synoviocytes and macrophages. The sublining layer is made of connective tissue containing blood vessels, fibroblast, adipocytes, and some resident immune cells like macrophages and mast cells [98]. During chronic inflammation the intimal lining layer is characterized by marked hyperplasia, whereas the sublining layer is the site of massive infiltration of immune cells (**Figure 1.9**).

RA is also characterised by the presence of circulating autoantibodies such as rheumatoid factor (RF) and anti-citrullinated peptide/protein antibodies (ACPA), strongly suggesting an autoimmune pathogenesis.

1.3.2 Classification criteria for rheumatoid arthritis.

The first classification criteria used to define RA were the 1987 American College of Rheumatology (ACR) criteria [99]. Although well accepted, these criteria had a significant limitation since they were helpful to discriminate only between patients with already established RA from those with other rheumatological diseases [100]. Therefore, in 2010 the American College of Rheumatology and the European League Against Rheumatism introduced the 2010 ACR/EULAR RA classification criteria in order to facilitate the study of patients at early stage of disease [100]. The new criteria give a score for each patient between 0 and 10. If the total score based on four different observations is 6 or higher the patient is classified unequivocally as an RA patient as long as the patient show at least one joint with synovitis (swelling) that cannot be explained by a different disease [100]. The four observations include (I) joint involvement, (II) serology, (III) acute phase reactants, and (IV) duration of arthritis (**Table 1.2**).

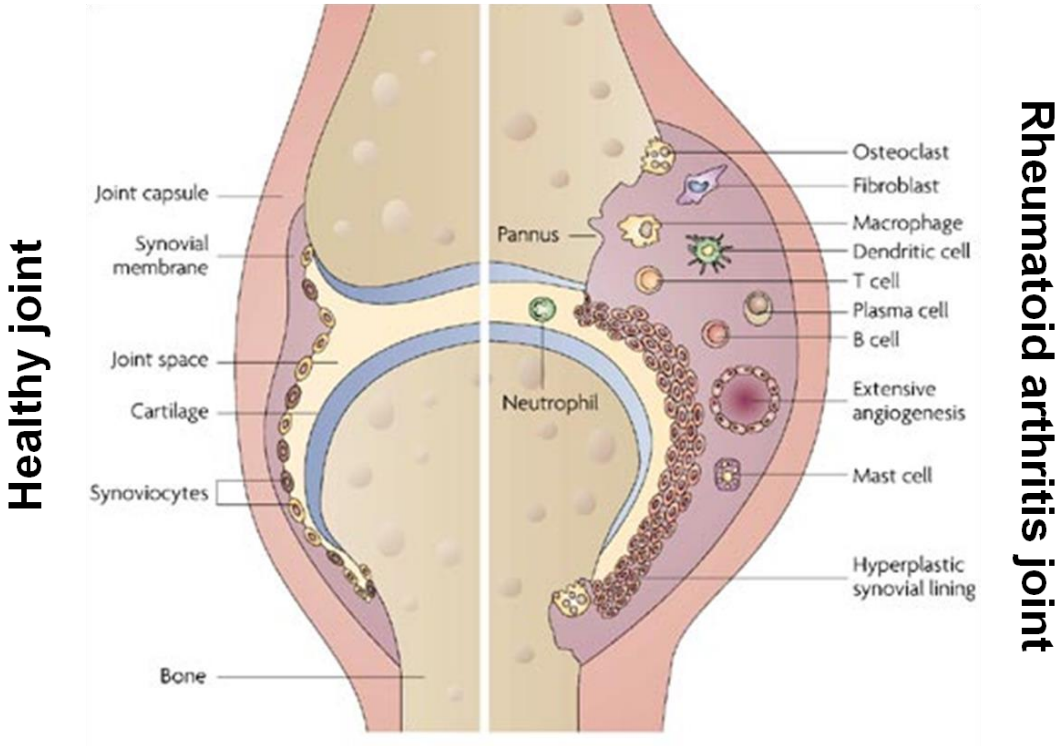

Figure 1.9 Normal and rheumatoid arthritis (RA) joint.

Schematic structure of a healthy joint (left) and rheumatoid arthritis joint (right) is shown. The RA joint is characterised by marked hyperplasia and by massive infiltration of immune cells.

Source: adapted from Strand et al. Nature Reviews Drug Discovery (2007)

Classification Criteria for RA	
A total score of 6≥10 is needed for classification as RA patients	**Score**
1. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints	2
4-10 small joints	3
> 10 joints	5
2. Serology	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
3. Acute-phase reactants	
Normal C-reactive protein (CRP) and normal erythrocyte sedimentation rate (ESR) 0	0
Abnormal CRP or normal ESR 1	1
4. Duration of symptoms	
<6 weeks	0
≥6 weeks	1
Clinical practice	
• ≥2 swollen joints	
• Morning stiffness lasting more than one hour for at least 6 weeks	
• Detection of RF and ACPA autoantibodies	

Table 1.2 Classification criteria for rheumatoid arthritis [100].

1.3.3 Aetiology.

The aetiology of RA is unknown. However, as seen for Sjögren's syndrome in Chapter 1.2 and other autoimmune diseases, a combination of genetic, environmental factors, gender and immunological alterations has been implicated in the development of RA.

1.3.3.1 Genetic background.

In RA the most relevant genetic risk factor is the class II MHC haplotype of an individual. In particular, certain human leukocyte antigen (HLA)-DRB1 alleles (HLA-DRB1*01 and HLA-DRB1*04) that contain a common amino acid motif (QKRAA), known as shared epitope, have been strongly associated with susceptibility to develop RA, mainly in patients who are positive for RF or ACPA [96, 101]. Regarding non-HLA genes, among others, single nucleotide polymorphisms have been identified in loci related to immune regulation, involving nuclear factor κB (NF-κB)-dependent signalling (e.g., TRAF1-C5 and c-REL) and T-cell stimulation, activation, and differentiation genes (e.g., PTPN22 and CTLA4) [96], mostly in ACPA-positive patients.

1.3.3.2 Environmental factors.

Smoking increases the risk of RA mainly in people with susceptibility HLA-DRB1*04 alleles. Citrullinated peptides have been detected in smoker RA patients, and this could induce the generation of ACPA in susceptible individuals [102]. Moreover, infectious agents (e.g. Porphyromonas gingivalis, Epstein-Barr virus, cytomegalovirus, Escherichia coli, Chlamydia and several other pathogens) have been linked with RA [96]. It has been proposed that the pathogen could start the disease through several mechanisms,

such as direct infection of the synovium, activation of innate immunity by TLRs activation, or molecular mimicry.

1.3.3.3 Gender.

RA is also predominant in women with a female:male ratio of 3:1. It has been proposed that the hormone milieu (i.e., the role of estrogens) can significantly influence some cells which are known to participate in RA pathogenesis. In particular, it has been shown that autoantibody-producing B cells are more resistant to apoptosis when they are exposed to estradiol, and that the fibroblast-like synoviocytes which express the estrogen receptor can increase the production of metalloproteinase when stimulated with estrogens [103]. Finally, one interesting finding is that during pregnancy RA patients often improve; although the mechanism of protection is not well defined. It is believed that a shift from Th1 to Th2-mediated immunity may play a role.

1.3.4 Extra-articular manifestations of rheumatoid arthritis.

RA is a systemic autoimmune disease. Thus, besides the joints, RA can affect many tissues and organs. A summary of the main systemic effects associated with RA is reported below.

- Patients with RA are characterised by an increased risk of cardiovascular diseases, i.e. atherosclerosis, myocardial infarction, and stroke [104, 105]. Inflammatory mediators including cytokines (e.g., IL-6 and TNF-α), immune complexes, and altered lipid particles are believed to be responsible for the increased cardiovascular risk in RA patients [96].

- Inflammation in patients with RA can also affect the brain, liver, lungs, exocrine glands (secondary SS), muscles, and bones (osteoporosis) [96].
- The risk of lymphoma is enhanced in patients with RA and is strongly associated with inflammation [106, 107].
- Patients with RA are characterised by higher risk of developing lung cancer. The association between smoking and RA is a likely explanation although also inflammatory mediators may have an important role in conferring the increased risk of lung cancer [96].

1.3.5 B cell in rheumatoid arthritis.

Several studies in the field of applied immunology, in particular focusing on synovial immune abnormalities, together with novel biological therapies targeting specific pathways have facilitated considerable progresses in understanding the pathogenesis of RA [108]. As outlined above, the hallmark of RA is infiltration of synovial tissue with mononuclear cells, mainly T cells, macrophages and B cells, together with synovial lining layer hyperplasia. In this paragraph, the role of B cells in the pathogenesis of RA is discussed.

A pathogenic role for B cells has been confirmed without any reasonable doubt by the efficacy of rituximab (RTX) in the treatment of RA [96]. RTX is a chimeric monoclonal antibody formed by human IgG1 and kappa constant region and by mouse variable region. RTX binds the B cell surface antigen CD20, a B cell-specific calcium channel expressed in all B cell subsets except pro-B cells and plasma cells [109]. Since pro-B cells do not express CD20, after treatment with RTX the bone marrow can start to

repopulate B cells. Although effective in up to 60% of RA patients, the remaining 40% does not respond and most of the responsive patients relapse within 6-8 months after treatment. The mechanisms of resistance/relapse to RTX are currently unknown and are not directly dependent on peripheral B cell depletion, which is achieved in all cases. In particular, it is unclear whether disease relapse might be determined by B cell repopulation of the synovial membrane and whether this repopulation comes from the systemic circulation or from “escaped” clones surviving in “protected” synovial niches.

In any case, efficacy of B cell depletion in RA has placed B cells at the centre stage in RA pathogenesis; thus understanding the mechanisms leading to their activation, particularly in the RA synovial tissues, is of critical importance.

In approximately 30% of RA patients, the rheumatoid synovial tissues have been shown to acquire features of ectopic GC, such as follicular dendritic cell (FDC) network, T/B cell segregation and high endothelial vessels (HEV) formation [13, 110]. As seen for SS, these ectopic lymphoid structures (ELS) are functional tertiary lymphoid organs capable of sustaining the molecular machinery necessary for SHM and CSR [111]. This demonstration, confirmed when RA synovium is transplanted into SCID mice [(*)], shows a capacity for self-sustained proliferation of B cells and local autoantibody production in the absence of re-circulating immune cells. Although it is still unknown whether the same or different antigen(s) (Ag) drive the production of autoantibodies in the RA synovium vs SLOs, these findings support a direct pathogenic role for ELS in promoting/maintaining autoimmunity in RA. Furthermore, their presence may also explain why drugs that effectively clear B cells from the bloodstream, such as RTX, do not always produce a marked clinical improvement in RA.

(*) SCID mice
Severe Combined Immunodefinciency (SCID) mice are routinely used as a model to study the biology of the immune system since they are impaired in the ability to make T and B lymphocytes or activate components of the complement pathway. Therefore, these mice are not able to fight infections, reject tumors and transplants.

1.3.5.1 Abnormalities in circulating B cell subpopulations in RA patients.

In RA the absolute number of circulating CD19+ B cells is similar to healthy individuals. However, patients with RA are characterised by a significant reduction in circulating CD27+ memory B cells and by a normal distribution of CD27- naïve B cells and early plasma cells [76]. In particular, patients with very early arthritis (i.e., less than 6 weeks after arthritis onset) display a selective reduction in CD27+IgD+ unswitched memory B cells [112], whereas patients with established RA are characterised by a reduction in circulating CD27+IgD- memory switched B cells [113]. Although no direct demonstration has been provided, the observed reduction in the RA memory B cell compartment has been explained as result of migration of these cells into the inflamed synovium. Despite the above evidence supporting that altered frequency of B cell subpopulations are a feature of RA, more studies are needed in order to clarify (i) whether these abnormalities represent primary pathogenic events or are secondary to the establishment of a chronic inflammatory process and (ii) whether they can predict disease evolution and response to treatment.

1.3.5.2 Autoantibodies in rheumatoid arthritis.

The first evidence of the autoimmune nature of RA came with the discovery of RF in the blood of patients. Along with RF, several autoantibody specificities have been described in RA, but only anti-citrullinated protein antibodies (ACPA) have been demonstrated of clinical utility and are currently used in clinical practice. However, autoantibodies titers normally do not correlate with disease activity although their production precedes the onset of clinical arthritis by many years, suggesting that breach of B self-tolerance is an early event in RA [114].

1.3.5.3 Rheumatoid Factor.

RF is an autoantibody directed against the Fc portion of the immunoglobulin (Ig), either IgG or IgM, although classical RF are IgM directed against IgG. Patients seropositive for RF are generally characterised by a more severe clinical disease compared to seronegative patients. RF seems able to fix and activate the complement locally in the rheumatoid synovium [115], thus contributing to synovial inflammation. IgG and IgM RFs are likely pathogenic in RA and large quantities of IgG can be found in the rheumatoid synovial tissue. However, there is also evidence of IgE and IgA RF in some patients with RA. In RA RFs are produced by B cells characterised by the presence of somatic mutations as consequence of affinity maturation [116], whilst they are often encoded by germline Ig genes in healthy individuals.

1.3.5.4 Anti-citrullinated protein/peptide antibodies (ACPA).

Although RF is detected in the majority of RA patients, it is not highly specific for RA as it is found in other chronic inflammatory conditions as well as in healthy individuals in

the late stage of their life [117]. However, detection of RF is still included in the classification criteria, as listed above. Recently, an increased interest in antibodies directed against citrullinated peptides which seem to have a better prognostic implication, has been seen in patients with RA.

In 1964 the first ACPA was reported in patients with RA as the anti-perinuclear factor (APF) and later as anti-keratin antibodies (AKA) [117, 118]. Later, it was shown that both APF and AKA are directed against the epithelial intermediate filament associated protein filaggrin which is involved in the aggregation of cytokeratin filaments during cornification of the epidermis. Subsequently, filaggrin citrullination was demonstrated as an essential step in its immunogenicity and cyclic citrullinated peptides has since been used as antigens in ELISA testing to detect ACPA.

More recently, antibodies directed against citrullinated keratin, vimentin, fibrinogen and xenoproteins as EBV-derived peptides were found in RA patients [119]. ACPA are currently the most specific autoantibodies for RA, although a few healthy individuals (about 2%) have been found positive for ACPA. Some patients with systemic inflammatory diseases like SLE and SS can have ACPA as well [117].

Citrullination of proteins is a physiological process that can occur also during inflammation and is therefore not specific for RA. However, an aberrant B cell response against citrullinated antigens may be specific for RA [117, 120]. It is clear that ACPA are part of the unique pathogenesis of RA, although it is not known if these antibodies are just a manifestation of an early inflammatory process or play a causative role [117].

1.3.5.5 Other potential autoantigens in RA.

The number of potential joint-specific and non-specific antigens that might have a role in the pathogenesis or aetiology of RA is extensive and to date there is no evidence of one specific RA antigens driving the disease, particularly in the synovial microenvironment. Cartilage-specific antigens include type II collagen, the cartilage glycoprotein gp39, proteoglycans, aggrecan, and cartilage link proteins. Among the non-articular antigens, autoantibodies against glucose-6-phosphate isomerase, heterogeneous nuclear ribonucleoprotein-A2, heavy chain binding protein, and heat shock proteins have been detected in the sera of some RA patients [121] but their diagnostic and/or pathogenic role is unclear.

1.3.6 Treatment of patients with RA.

Pharmacological treatments of RA patients are used to control pain and inflammation and prevent bone erosions with the aim to achieve remission or a low disease activity for all patients. The treatments commonly used include corticosteroids, oral disease-modifying antirheumatic drugs or DMARDs, and biological DMARDs (anti-TNF drugs as adalimumab, etanercept, infliximab; and others like abatacept, anakinra, rituximab, and tocilizumab) [122].

- **Corticosteroids.** Corticosteroids derive from cortisol which is a hormone produced by the adrenal glands. They are used for their anti-inflammatory and immunosuppressive properties since they can inhibit recruitment of inflammatory cells to the site of inflammation, neutrophil function and prostaglandin production. They are commonly administrated orally and the corticosteroids mainly used are

methylprednisolone, prednisone, and prednisolone. Their current use is primarily as intra-articular or intra-muscular injections or as short-term oral treatments.

- **Oral disease-modifying antirheumatic drugs (DMARDs).** Oral DMARDs act modifying the course of inflammation. The aim of these drugs is to reduce or prevent joint damage, and preserve function and structure during the inflammatory phase. They include hydroxychloroquine, leflunomide, methotrexate (MTX), and sulfasalazine.

- **Biological DMARDs.** Biological DMARDs are normally given by injection and their targets are component of the immune system (i.e., cytokines as TNF, IL-1, and IL-6; or immune cells as B cells).

Anti-TNF drugs. Adalimumab, certolizumab pegol, etanercept, golimumab, and infliximab are all anti-TNF drugs licensed for their use in RA. Adalimumab, golimumab, certolizumab pegol and infliximab are monoclonal antibodies which target TNF. In particular, adalimumab is a fully human monoclonal antibody that binds to TNF, blocking its interaction with the TNF receptor. Golimumab is a human monoclonal antibody which binds TNF-α with high affinity. Infliximab is a human/mouse chimeric monoclonal antibody that binds also human TNF-α. Certolizumab pegol is a PEGylated Fab' fragment against human TNF that lacks the Fc region. Etanercept is a TNF soluble receptor protein linked to the Fc portion of IgG1. It binds circulating TNF, thus blocking its interaction with the receptor.

Other biologics. Rituximab is a chimeric monoclonal antibody that binds CD20 expressed by B cells and plasma blasts, thus removing almost all the B cells from the periphery with the exception of pre-B cells and plasma cells which do not express CD20. **Tocilizumab** is a monoclonal antibody that blocks IL-6 receptor, thus blocking IL-

6 action. **Anakinra** is a IL-1 receptor antagonist. Finally, **Abatacept** is a soluble fusion protein (CTLA-4 with the modified Fc portion of IgG1) that interferes with T cell activation.

1.4 Ectopic lymphoid neogenesis.

1.4.1 General aspects.

The adaptive immune system response to antigens including tolerance induction, antigen-specific immunity and immunological memory [123] is, in most cases, supported by SLOs which in humans include lymph nodes (LNs), spleen and mucosal associated lymphoid tissues (MALT).

Tertiary lymphoid organs, also known as ectopic lymphoid structures (ELS), form at the site of chronic inflammation through a process known as lymphoid neogenesis [77],[124]. Immunohistochemical analysis performed on the target tissues from patients with autoimmune and infectious diseases show that lymphoid neogenesis is a dynamic process during which lymphocytic infiltrates develop into aggregates that can eventually organize in secondary B-cell follicles with GCs and T cell compartments containing DCs and high HEVs [13] through which lymphocytes can enter from the systemic circulation [124].

The cellular constituents of B- and T-cell zones in SLOs and ELS are similar. ELS include proliferating B cells and FDC networks which are essential for B cell maturation and to stimulate proliferation and prevent apoptosis of GC B cells [125, 126]. Furthermore, naïve B cells, centroblasts, centrocytes, memory B and plasma cells, T cells are all detected in ELS or in the nearby biological fluids. However, some elements differ markedly from that of classical SLOs. Unlike LNs, but similar to MALT, ELS are characterised by the absence of afferent lymphatic vessels and are not encapsulated,

which means that they are directly exposed to antigens and cytokines from the inflamed environment [13].

The role of ELS is not entirely clear, in particular whether they play a beneficial or a detrimental function. This may depend on both the site of inflammation and the stimuli that are involved in their induction. Furthermore, it is not clear why these structures should form in the periphery in living organisms. It has been hypothesized that the formation of these immunologically active structures may take place to avoid persistent activity in critical SLOs and prevent systemic dissemination of (auto)antigens, or to maximize antigen presentation at the local site of inflammation which in turn would increase efficiency of the immune response through the concentration of cells, cytokines and immunoglobulin in a specific location [108]. Thus, when ELS develop at site of pathogenic infection, they can help to eliminate or neutralize pathogens by generating antibody-producing plasma cells specific for the pathogen. However, when ELS develop at sites where autoantigens are continuously being exposed, they may lead to the activation of autoreactive lymphocytes and, eventually, induce tissue destruction [124]. Evidence of ELS formation has also been obtained in a number of chronic infectious diseases. For instance, *Helicobacter Pylori*-induced gastritis is often accompanied by lymphoid tissue neogenesis leading to the production of plasma cells producing IgA antibodies specific for *H. Pylori*-associated antigens [13, 127, 128]. Lymphoid neogenesis has been observed also in HCV-mediated chronic hepatitis and protozoa-induced pathologies (placental malaria) [13]. Aside from chronic infection, ELS are also induced in several autoimmune diseases, such as type I diabetes, Hashimoto thyroiditis, myasthenia gravis, multiple sclerosis, and as it

will be described in this chapter, RA and SS [13, 128, 129]. Organized lymphocytic infiltrates have been observed in murine models of autoimmunity such as spontaneous diabetes in NOD mice and autoimmune gastritis [128, 130-132], and also during graft rejection. Tissues undergoing chronic graft rejection are characterised by HEV-like venules, T cells, B cells producing anti-graft antibodies, while ELS have also been reported in rejected human allografts [128, 133, 134]. Lastly, very few data on lymphoid neogenesis in cancer have been reported supporting the hypothesis that ELS formation is somewhat impaired in malignant conditions, possibly resulting in a less efficient immune response against neoplastic cells [13]. In this regard, recent evidence suggest that exploitation of lymphoid chemokines, which are critical regulator of ELS formation (see Section 1.4.2 below), might be able to drive lymphocyte accumulation and induce lymphoid neogenesis at tumor sites as a form of cancer immunotherapy [128].

1.4.2 Role of lymphoid chemokines in lymphoid organ development and organization.

The cascade of events that lead to ELS formation in pathological conditions shows several similarities with the signalling pathways involved in secondary lymphoid tissue organogenesis which is the result of a highly coordinated interaction between haematopoietic cells, non-lymphoid stromal cells, adhesion molecules, chemokines, cytokines, and growth survival factors [13]. Thus, in order to understand the development of ELS, it is essential to briefly review the biological basis that lead to

SLOs formation giving particular emphasis, in this context, at the active role of lymphoid chemokines in this process.

1.4.2.1 Role of lymphoid chemokines in SLOs development.

SLOs are highly specialised structures, which develop in order to regulate leukocyte trafficking and recirculation at dedicated sites and thus optimize the generation of an adaptive immune response following an antigenic challenge. As previously outlined, leukocyte trafficking within LNs is a result of a tightly regulated process which involves stromal and vascular anatomical structures and chemoattractant gradients. The chemoattractant signalling pathways involve several "lymphoid" chemokines, such as B-cell activating chemokines-1 (BCA-1)/CXCL13, Epstein-Barr virus-induced gene 1 ligand chemokines (ELC)/CCL19, secondary lymphoid tissue chemokines (SLC)/CCL21 and stromal cell-derived factor-1 (SDF-1)/CXCL12 and their specific receptors CXCR5 (for CXCL13), CCR7 (CCL19 and CCL21) and CXCR4 (for CXCL12). In particular, the trafficking of $CCR7^+$ naïve and central memory T cells and mature dendritic cells (DCs) is regulated by CCL19/CCL21 and peripheral node addressin ($PNAd^+$) HEVs. CCL19 and CCL21 are produced by stromal reticular cells [135] within the T-cell rich area of the LN. The concomitant presence of CCL19/CCL21 and HEVs allows the homing of T cells and mature DCs to SLOs from the systemic circulation upon binding to $PNAd^+$ HEVs through L-selectin and to position themselves in close association with fibroblastic reticular cells where T cells receive a strong survival signal mediated by IL-7 [135]. Although in lower amount, also naïve B cells express CCR7 and, thus, together with CXCR4 they use this receptor to enter SLOs from the systemic circulation [136].

However, it is CXCR5 in response to a CXCL13 gradient (see below) that ultimately determines the positioning of B cells in specific B cell follicles. The organisation of B cells into follicles is regulated by lymphotoxin-$\alpha 1\beta 2$ ($LT\alpha 1\beta 2$) – a membrane bound heterotrimeric member of the tumour-necrosis factor (TNF)-family, and as mentioned above by CXCL13 [137]. $LT\alpha 1\beta 2$ can be expressed by B cells themselves, and upon ligation of the LTβ-R on resident follicular stromal cell induces CXCL13 production and further $LT\alpha 1\beta 2$ upregulation as well as the clustering of the B cells [137]. Within the GC, CXCL13 production is sustained by FDCs. CXCL13 mainly directs GC B cells to the light zone of the GC where antigen selection takes place. Within the GC, another homeostatic chemokine, CXCL12, is critically involved in the migration of $CXCR4^{high}$ centroblasts to the dark zone where SHM takes place.

CXCL13 is also involved in the initiation of events that lead to lymphoid organs formation. Indeed, mice lacking CXCL13 and its receptor CXCR5 show a partial blockage of LN organogenesis [137].

Thus, in addition to the functional role in the organization of lymphoid organs in adults, the lymphoid chemokines are extensively involved in the generation of SLO during lymphoid organogenesis in prenatal life. In particular, lymphoid chemokines are produced pre-natally by $VCAM\text{-}1^{+}ICAM\text{-}1^{+}LT\beta R^{+}$ embryonic mesenchymal “organizer” cells in response to close interaction with $CD3^{-}CD4^{+}CD45^{+}IL\text{-}7R\alpha^{+}RANK^{+}$ embryonic hematopoietic “inducer” cells which also express CXCR5. A further key signal necessary for lymphoid organ development is sustained by two members of the TNF family, LTα and LTβ. LTα can exist either as a soluble homotrimer ($LT\alpha 3$) or as heterotrimer ($LT\alpha 1\beta 2$). The binding of $LT\alpha 1\beta 2$ to its receptor LTβ-R expressed on stromal cells

induces the expression of CXCL13, CCL19 and CCL21, and HEVs formation [128]. The non-redundant and upstream role of lymphoid chemokines CXCL13, CCL21 and CCL19 in promoting lymphoid tissue organization has been clearly demonstrated *in vivo* in transgenic animals, whereby overexpression of these factors in the pancreas or thyroid induces, in a LTB dependent fashion, the formation of ELS recapitulating several feature of lymphoid organ development [108, 138-140].

Thus, lymphoid chemokines exert a critical role during lymphoid organogenesis, allow the transformation of diffuse infiltrates into highly organized structures which are necessary for the initiation of the adaptive immune response, and importantly play an important part in ELS development, although they are not sufficient to drive the complete formation of lymphoid structure [13].

1.4.2.2 Role of lymphoid chemokines in ELS formation.

It has been postulated that lymphoid chemokines exert a similar role in regulating ELS formation and organization, which will be reviewed in the next paragraphs together with the potential role of lymphoid chemokines in supporting functional niches of autoreactive B cells, in the target organs of two clinical conditions, the synovium of RA patients and the salivary glands of patients with SS.

1.4.3 Ectopic lymphoid neogenesis in rheumatoid arthritis and Sjögren's syndrome.

It is now clear that a subset of patients with RA (around 40%) and SS (around 30%) develops ELS in the synovium and salivary glands, respectively. These structures are characterised by aggregated of T and B cells often showing T/B segregation,

development of HEVs and FDC networks. Among different patients, inflammatory infiltrates are characterised by different degrees of structural and immunological organization. Therefore, my laboratory has recently adopted a histomorphometric analysis based on the aggregate radial cell count which has led to the development of the following grading system: grade 1 (G1) define the first stage of the lymphocytic aggregation mainly characterised by $CD3^+$ T cells; grade 2 (G2) are normally enriched in the $CD20^+$ B cell component and characterised by a higher level of organization; and grade 3 (G3) define larger aggregates with often T/B cell compartmentalization and the presence of FDC network [108, 141, 142].

Evidences in patients with and animal models of RA and SS have demonstrated that the formation and maintenance of these ELS is critically dependent on the ectopic expression of lymphotoxins (LT) and homeostatic chemokines CXCL13, CCL19, CCL21 and CXCL12. These key molecules are produced during chronic inflammation by several cell types, including resident epithelial (in SS, see below), stromal and endothelial cells as well as different subsets of infiltrating immune cells.

Briefly, chemokines can have both homeostatic and inflammatory properties. As previously reported, the homeostatic chemokines are primarily involved in lymphoid neogenesis, though it has been show that they may also be involved in inflammation [143].

1.4.3.1 Role of lymphoid chemokines in rheumatoid arthritis.

As previously reported, among the factors involved in ectopic lymphoid neogenesis, the lymphoid chemokines CXCL13 and the CCR7 ligands CCL21 and CCL19 play a central

role in this process. The activation of molecular pathways similar to the mechanisms of SLO organogenesis has been demonstrated in RA by gene expression studies showing that CXCL13, CCL19 and CCL21 can be upregulated in the rheumatoid synovium. The increased level of lymphoid chemokines is also associated with enhanced levels of LTβ, of genes associated with IL-7 signalling pathway, and with the presence of ELS [108, 111, 138, 144]. Studies on the relationship between chemokine expression and the different degree of lymphoid organization in RA synovial tissue have shown that CXCL13 and CCL21 can be synthesized both in highly organized structures with FDC network, T and B cell segregation, but are also expressed, although at lower levels, in smaller and less organized lymphoid follicles. Based on this evidence, the current working hypothesis is that lymphoid chemokines are regulated upstream of ELS organization [145].

My laboratory and other groups showed that in the rheumatoid synovium, in addition to FDC networks, the homeostatic chemokine CXCL13 is mainly produced by hematopoietic cells such as $CD14^+/CD68^+$ monocyte/macrophages [146] and a subset of memory T cells [147], suggesting that cells of both the innate and adaptive immune system in the synovial microenvironment play an important role in the recruitment and organization of B cells [148]. Importantly, the expression of CXCL13 is closely associated with the formation of B cell-rich aggregates with features of ELS. In keeping with an essential role of CXCL13 in ELS formation in the RA synovium, its receptor CXCR5 has been shown to be widely expressed by infiltrating B cells and subsets of T cells [143].

In contrast to CXCL13, CCL21 is mainly expressed in the T cell-rich areas of synovial G3 lymphoid aggregates and in close association with HEVs co-expressing PNAd. Although closely associated with vascular structures, the expression of CCL21 is confined to smooth muscle actin $(SMA)^{+}$ $CD45^{-}$ myofibroblast-like stromal cells, similarly to what has been observed in human and murine SLOs [108]. Overall, this demonstrates that in the RA synovium the lymphoneogenetic programme is associated with the differentiation of stromal cells with the capability of supporting the recruitment of $CCR7^{+}$/L-$selectin^{+}$ naïve and central memory T cells and $CCR7^{+}$ mature DCs.

1.4.3.2 Role of lymphoid chemokines in Sjögren's syndrome.

Several studies on the role of lymphoid chemokines in the organization of ELS have been performed also in the salivary glands of SS. Similar to RA, our group has shown that the expression of CXCL13 and CCL21 is progressively increased with the gradual acquisition of lymphoid features in the salivary glands. Indeed, both lymphoid chemokines were found to be expressed already in G1 clusters as well as G2 aggregates in the absence of T/B cell compartmentalization. Analysis performed on the identification of the cellular source of lymphoid chemokines in the target organ of SS have revealed that both resident, non-lymphoid cells as well as infiltrating immune cells can produce lymphoid chemokines. In particular, Xanthou et al showed that ductal epithelial cells and infiltrating mononuclear cells were the main source of lymphoid chemokines in SS [149]. The presence of lymphoid chemokines on epithelial cells were confirmed by Salomonsson et al who showed expression of both CXCL13 and CCL21 in acinar and ductal epithelial cells. These data would be in keeping with the

important role of epithelial cells in the pathogenesis of SS (also described as "autoimmune epithelitis")which are known to actively contribute to the inflammatory process in the salivary glands through production of several inflammatory chemokines and cytokines. Interestingly, epithelial expression of CXCL13 and CCL21 was not found to correlate with the development of ELS or the size of periductal lymphocytic foci in SS salivary glands [150]. In contrast with the hypothesis that epithelial cells are the main source of lymphoid chemokines in SS, Amft et al reported expression of CXCL13 mainly in endothelial cells in blood vessel and networks of stromal cells resembling FDCs within the inflammatory foci, while ductal epithelial cells displayed primarily high levels of CXCL12 [151]. Studies performed by my laboratory confirmed that while CXCL12 was strongly expressed by ductal epithelial cells, CXCL13 was only weekly expressed by epithelial cells, being primarily produced by infiltrating immune cells as well as networks of stromal cells within the inflammatory foci [152]. Importantly, we observed that only immune cell- but not epithelial-derived CXCL13 was associated with the development of ELS in the salivary glands of SS patients.

Similar to RA, my laboratory also showed that within the subsets of infiltrating immune cells the homeostatic chemokine CXCL13 was mainly produced by $CD68^+$ macrophages and $CD3^+$ T cells [152]. Interestingly, high levels of CXCL12 were also observed in a subset of $CD68^+$ cells resembling tingible body macrophages within ELS [152]. Furthermore, again in keeping with similar data in the RA synovium, CCL21 was mainly expressed by HEV-associated stromal cells in the salivary glands developing preferentially in the T cell areas of segregated lymphocytic foci [152].

In addition to chronic inflammation in SS, my lab recently showed that lymphoid chemokines also exert an additional role in the development of extranodal B cell lymphomagenesis and particularly in salivary gland MALT lymphoma (MALT-L), which develop in around 5% of SS patients. Interestingly, CXCL13 was found to be expressed by malignant B cells in gastric MALT-L as well as in non-Hodgkin B cell lymphoma of the central nervous system where also CXCL12 and its receptor CXCR4 were found to be expressed [127, 153, 154]. These studies, performed on a large cohort of SS parotid glands with lymphoepithelial sialoadenitis (LESA) and MALT-L, have shown that CXCL13 and CCL21 were expressed at higher levels in SS salivary glands with LESA as compared to MALT-L. Conversely, CXCL12 was more expressed in parotid glands with MALT-L in comparison to LESA [152]. Interestingly, the expression of CXCL13 and CCL21, sustained mostly by macrophages and T cells (for CXCL13) and stromal cells (for CCL21), was found to be associated with reactive areas populated by $CD20^{+}IgD^{+}Bcl\text{-}2^{+}Bcl\text{-}6^{-}$ follicular B cell and $CD20^{+}IgD^{-}Bcl\text{-}2^{-}Bcl\text{-}6^{+}$ GC B cell associated with FDC networks. Conversely, CXCL12 was mainly associated with and often produced by malignant $CD20^{+}IgD^{-}Bcl\text{-}2^{+}$ marginal zone-like B cells [152].

Hence, we suggest that CXCL13 and CCL21 can directly control the formation and maintenance of functional ELS in SS salivary glands which in turn can promote in a subset of patients the progressive development of B cell lymphomagenesis [152].

1.4.4 Functionality of ELS in RA and SS: role of activation induced cytidine deaminase.

A long outstanding question regarding ELS in chronic autoimmune conditions has been whether these structures simply recapitulate some cellular and molecular features of SLOs or whether they also represent ectopic niches sufficient to promote B cell functional activation. In this regard, my laboratory recently provided direct evidence of the capability of ELS to support activation and differentiation of B cells both within the synovial tissue of RA joints and in the salivary glands of SS patients. In both conditions lymphoid aggregates characterised by FDC network formation (as detected by immunohistology for CD21 or by expression of CD21 long isoform mRNA) invariably express AID at both mRNA and protein level [73, 111]. Importantly, AID expression can also take place in the absence of the full compartmentalization of the GC into dark and light zones which often differentiates ELS from SLOs. This work supported previous evidence both in RA and SS which demonstrated, by performing analysis of the V-gene repertoire from follicular structures microdissected from RA synovium and SS salivary glands, that a significant proportion of infiltrating B lymphocytes display an oligoclonal repertoire with highly mutated V regions, indicating a local antigen-driven process [72, 155-157].

In addition to SHM, my laboratory recently provided evidence that B cells also actively undergo class switching in the presence of ELS within the RA synovium [111]. Interestingly, it has been showed that Iγ-Cμ circular transcripts, which are specific transcribed by-products of CSR from IgM to IgG, are continuously expressed in synovial tissues engrafted into SCID mice, demonstrating that CSR can be sustained for several

weeks within the RA synovium characterised by $AID^+/CD21L^+$ lymphoid structures even in the absence of recirculating immune cells.

Overall, the conclusion is that ELS developing in the RA synovium and SS salivary glands activate the molecular machinery to sustain in situ Ig V repertoire diversification, isotype switching, B cell differentiation and oligoclonal expansion. It is important to note that this capacity is not an exclusive feature of lymphoid neogenesis in these conditions as similar evidence of AID expression in ELS has also been demonstrated in multiple sclerosis [158], and in chronic graft rejection [159] suggesting that chronic antigen exposure, driven by variable antigens in different target organs, sustain in situ B cell differentiation.

1.4.5 Contribution of ELS to organ specific autoimmunity in RA and SS.

One of the hallmarks of RA and SS is the presence of circulating auto-antibodies (Ab), as described earlier in this thesis. Thus, another important aspect to consider regarding ELS in autoimmune diseases in general and in RA and SS in particular is whether these structures directly contribute to breach of tolerance and autoimmunity over and above SLOs by promoting selection of autoreactive B cells, plasma cell differentiation and in situ autoantibody production.

In RA there is now clear evidence that the presence of ELS closely correlate with synovial plasma cell number, suggesting in situ differentiation within the GCs [160-162]. A clear confirmation of this possibility has been recently provided by the analysis of the Ig V gene repertoire and clonal relationship between ELS and plasma cells

isolated from the synovial tissue using laser capture microdissection. Using this approach the Berek's group provided the first direct demonstration that most synovial plasma cells are generated from locally activated B cells which then accumulate and migrate within the inflamed tissue [163].

In keeping with these observations, my group recently demonstrated that ectopic lymphoid follicles in RA patients are surrounded by $CD138^+$ plasma cells, some of which produce antibodies directed against the citrullinated, but not the unmodified, form of fibrinogen [111]. In addition, the use of the HuRA-SCID mouse model allowed to directly study in vivo the production of human autoantibodies derived from the synovial tissue without confounding production from SLOs and long lived plasma cells in the bone marrow.

By transplanting RA synovial tissues characterised by the presence or absence of ELS, my group demonstrated that AID^+CD21L^+ RA synovial grafts produce human IgG ACPA which can be measured at high levels and for several weeks in the mouse circulation. This suggests that in RA ELS can form self-sustained niches of autoreactive B cells which can differentiate into plasmablast/plasma cells and produce disease-specific autoantibodies within the target organ.

Similarly to RA, in SS there is compelling evidence that ELS participate to humoral autoimmunity within the salivary glands. Original evidence that SS salivary glands are characterised by the preferential accumulation and selection of autoreactive B cell clones derived from analysis of the Ig V repertoire of infiltrating B cells showing an abnormal distribution and preferential usage of Ig Vλ and Vκ genes, such as VκA27, frequently displayed by autoreactive B cells [164, 165]. In support of this initial

observation, Salomonsson et al later showed that plasma cells reacting against the ribonucleoprotein Ro/SSA and La/SSB preferentially accumulate around ELS in labial SG biopsies of SS patients, strongly suggesting that also in SS ELS support in situ differentiation of autoreactive plasma cells against disease-associated antigens [150].

An interesting concept regarding the formation of ELS was recently raised by Le Pottier et al demonstrating that, differently from GC in SLOs, ELS arising in the salivary glands of SS patients do not exclude autoreactive B cells which are thus capable of entering FDC networks, express AID and undergo a GC response [166].

According to this hypothesis, ELS fail to exert the physiological peripheral checkpoints against autoreactivity which are normally active in SLOs and prevent self-reactive B cells from entering the GC and undergo affinity maturation. A plausible mechanism implicated in such defective regulation of autoreactive B cell activation in ELS is again related to the importance of lymphoid chemokines. In physiological conditions, potentially autoreactive B cells are excluded from entering B cell follicles in SLOs because they are induced to down-regulate CXCR5 and become less responsive to CXCL13 compared to normal B cells which compete for the follicular niche [167]. As a consequence, autoantigen-binding B cells are forced to accumulate at the boundary between the T and B cell zone of SLOs where they do not receive activatory signals, become anergic and die by apoptosis. Although the mechanisms regulating CXCR5 down-modulation in autoreactive B cells are not well understood, it is possible to speculate that within ELS during chronic inflammation these counter-immunoregulatory pathways normally active in SLOs are deficient, resulting in the accumulation of autoreactive B cells within ectopic GCs.

Overall the above evidence strongly supports the conclusion that ELS in the RA synovium and SS salivary glands are functional and favour the selection, survival and proliferation of autoreactive B cells and their differentiation into auto-antibody producing plasma cells over and above SLOs. This process is critically dependent on chronic antigenic exposure within the target tissue and result in humoral autoimmunity against organ-specific antigens.

An important question, which remains unresolved, is whether ELS are important in the initial steps of breach of tolerance to self-antigens or, as it appears more likely, rather play a role in the perpetuation of autoimmunity and chronic inflammation.

Additionally, a fundamental question is which antigen(s) are responsible for driving such humoral autoimmune response within the target organs of SS and RA.

1.4.6 Interfollicular (IF) large B cells in SLOs and ectopic lymphoid structures.

As pointed in Chapter 1.1, in lymphoid organs AID expression is restricted to GC B cells and in particular to centroblasts which produce high levels of this enzyme [168]. In 2006, Moldenhauer et al demonstrated that outside the GC, a high percentage of AID+ B cells belonged to a newly characterised subset of B cells, called interfollicular (IF) large B cells, and were the only subset of B cells to express AID outside GC [169]. These cells, which were described already 20 years ago but never analysed thoroughly, were deeply characterised for the first time in the work by Marafioti and colleagues in 2003, using normal lymph nodes [170]. They showed that IF large B cells are a unique CD20+ B cell population which is distinct from the main established B cell subsets. These cells

are characterised by a large cytoplasm and are typically found in the interfollicular T cell rich area. They can carry mutated Ig genes, supporting AID functionality. The presence of somatic mutation also suggests that IF large B cells could be a new subset of post-GC B cells. IF large B cells lack detectable levels of the memory B-cell marker CD27, many of these cells are proliferating (positive for Ki-67), and about half of the cells express multiple myeloma oncogene protein 1 (MUM-1), a transcription factor found in plasma cells and a few (late) GC B cells but not in memory B cells. Arguments against a plasma cell identity of these cells include negativity for the plasma cell markers CD38 and CD138. Moreover, these cells are different from centroblasts (negative for Bcl-6) and centrocytes, the main two B cell subsets in the GC. The dendritic morphology and close association with T cells might also suggest a role in T cell activation, possibly via antigen presentation, although they lack co-stimulatory markers as CD80 and CD86. Interestingly, my group provided the first demonstration that the population of large IF B cells is not restricted to SLOs but can also be observed in the target organ of rheumatic autoimmune diseases, such as synovial tissue (for RA) [111] and salivary gland (for SS) [73]. These cells were the only B cell subset expressing AID outside FDC networks, though were only observed in the presence of ELS. Similar to SLOs, these cells are characterised by a large cytoplasm, by a dendritic-like morphology, and are localised in close association with T cells only outside CD21+ aggregates. However, the biological significance and the pathogenic role in autoimmunity and chronic inflammation as well as their phenotypic characterization in ELS have not been clarified as yet.

1.5 Immunoglobulin gene usage in rheumatic autoimmune diseases.

1.5.1 Introduction.

To date whether the Ig V gene usage in patients with autoimmune diseases differs from that of healthy individuals has been a matter of controversy. It has been proposed that the production of autoantibodies in autoimmune diseases could be a result of abnormalities in the Ig variable genes resulting in a preferential gene usage both for the H and L chain together with anomalies in receptor editing and in the number of somatic mutations. Therefore, the analysis of the Ig V gene repertoire has become of particular interest to understand whether the Ig gene usage is skewed in patients with autoimmune diseases compared to controls [171]. The analysis of productive and non-productive rearrangements in peripheral B cells in healthy individuals has revealed that the VH, Vκ and Vλ gene usage is naturally biased. Normally, among the seven VH gene families that are used to shape the Ig repertoire, VH3 which is the largest variable region family with 22 members, is used more frequently, followed by VH4 and VH1 in the productive repertoire. Similarly, in the Vκ and Vλ gene families there is a preferential family usage (**Table 1.3**) [171]. Analysis of the gene usage associated with autoantibodies has revealed no evidences for a preferential usage of VH, Vκ and Vλ family, expect for few autoantibodies. For instance, a marked increase of Vλ3 family usage has been reported for RF together with a specific restriction to genes of the VH1 and VH3 family [171]. There are also

indications that some autoantibodies, including RF, anti-dsDNA, Ro/SSA, La/SSB, phospholipids, histone A2, laminin, and collagen, use more frequently Vλ genes which are normally less expressed in the Ig repertoire of healthy individuals [171]. However, to date the number of Ig V genes analysed is too small to allow any comprehensible correlation between gene usage and autoimmune diseases.

A briefly overview of the analysis of the Ig repertoire in patients with SS and RA will be reported in the following section.

	Non-productive (%)	Productive (%)		Non-productive (%)	Productive (%)		Non-productive (%)	Productive (%)
VH1	4.0	13.1	**Vκ1**	47.0	44.0	**Vλ1**	25.4	29.1
VH2	8.0	1.9	**Vκ2**	29.0	19.0	**Vλ2**	30.9	33.1
VH3	36.0	53.9	**Vκ3**	13.0	29.0	**Vλ3**	5.4	15.7
VH4	44.0	24.8	**Vκ4**	6.0	5.0	**Vλ4**	18.2	5.8
VH5	4.0	2.9	**Vκ5**	4.0	2.0	**Vλ5**	7.3	3.5
VH6	4.0	2.4	**Vκ6**	1.0	< 1	**Vλ6**	3.6	3.5
VH7	0.0	1.0	**Vκ7**			**Vλ7**	1.8	4.1
						Vλ8	1.8	1.2
JH1	3.8	1.0				**Vλ9**	1.8	0.6
JH2	0	2.4	**Jκ1**	21.0	30.0	**Vλ10**	3.6	3.5
JH3	7.7	7.8	**Jκ2**	40.0	36.0			
JH4	50.0	55.3	**Jκ3**	4.0	6.0	**Jλ1**	5.5	7.0
JH5	15.4	9.7	**Jκ4**	20.0	17.0	**Jλ2/3**	34.5	39.0
JH6	23.1	23.8	**Jκ5**	15.0	11.0	**Jλ7**	60.0	45.1

Table 1.3. Family gene usage in peripheral CD5-/IgM+ B cells isolated from healthy individuals.

Source: Foreman et al., Autoimmun Rev. 2007 [171]*; Lipsky et al., J.Clin.Invest. 1997* [172]

1.5.2 Immunoglobulin repertoire analysis in SS and RA patients: a general overview.

Several studies on the Ig repertoire of B cells isolated from SS patients have been done by comparing peripheral blood and inflamed parotid tissue. The importance to study the Ig gene usage of B cells infiltrating the parotid is that it might provide a more specific scenario of local selection and antigen-dependent proliferation of B cells at the local site of inflammation [173]. In particular, Dorner and colleagues [173] have found no evidence of different distribution of VH families comparing blood and parotid of SS patients. The VH gene usage was also similar to that found in B cells isolated from peripheral blood of normal individuals. This suggests that the B cells in the inflamed parotid gland are a polyclonal population. In contrast, they have noticed that the majority of glandular B cells from SS patients have mutated VH rearrangements with a mutational frequency higher within the productive repertoire compared to B cells isolated from peripheral blood. Furthermore, they have found differences in CDR3 length, with glandular B cells showing shorter CDR3 region than the peripheral counterparts. They suggested that this may be due to a less usage of JH6 which among all the JH segments encodes the longest CDR3 [174]. This result confirms the observation that the JH6 segment is positively selected in the expressed pre-immune repertoire and negatively in the mutated repertoire [174].

In contrast to the Ig repertoire of the heavy chain, B cells from parotid gland of SS patients showed differences in the productive light chain repertoire with a preferential usage of certain Vκ and Vλ genes [165]. This observation may support the idea that

even though the B cells in the parotid represent a polyclonal population with an Ig heavy chain repertoire similar to healthy individuals, the local microenvironment may allow the clonal expansion and selection of B cells which express particular light chain genes [165]. Finally, it was shown that productive VL chain rearrangements of glandular B cells (in particular Vλ genes) are characterised by higher mutational frequency compared to peripheral blood [165].

Similar studies in peripheral B cells from RA patients have revealed no significant differences in the overall Ig VH gene usage compared to controls. Only a preferential usage of particular VH genes (i.e., V4-34, V3-23 and V1-69) has been associated with RF [175]. In particular, in RA patients an increase in somatic mutations has been observed in the CDRs suggesting an antigen-driven B cell selection in these patients. However, whether the increase of somatic mutations is associated with the pathogenesis of RA is still unclear [175]. An interesting study [176] has been done also comparing the Ig VH repertoire before and after treatment with RTX which has been shown to be beneficial for RA patients, as reported in Chapter 1.3. This study showed that at the baseline the overall Ig repertoire for the heavy chain was similar to the one expressed in healthy individuals with few differences in certain VH genes, already associated with autoimmune diseases. These included an over-expression of the VH 1-69 and VH 4-34 genes and a reduced usage of the VH 3-07 and VH 3-30 genes in RA compared to controls. The analysis of the Ig repertoire in the repopulated B cells after therapy (i.e., seven month after the first infusion of RTX) showed significantly changes in the VH, D and JH gene usage, suggesting a possible therapeutic modulation of the VH gene repertoire by RTX treatment [176].

1.5.3 Receptor editing.

Receptor editing is one of the three main mechanisms physiologically used to control autoreactive B cells. As explained in Chapter 1.1, this process refers to a secondary rearrangement in which the heavy chain combines with a new light chain. Studies of the Vκ and Vλ gene usage have revealed that receptor editing is defective in some patients with autoimmune diseases. For example, over expression of Jλ2/3 and a reduced frequency of Jλ7 was found in peripheral B cells of SS patients, suggesting a decreased occurrence of receptor editing [171]. Defects in receptor editing have been also observed in SLE patients where a highly skewed use of proximal Vκ and less use of downstream Jκ genes was found in anti-DNA antibodies of some SLE patients, whereas usage of Jλ proximal genes were decreased with an increase in the distal cluster in a patient with untreated SLE, suggesting an increase of receptor editing [171].

Chapter 2 | Rationale and Aims of the thesis

2 | Rationale of the thesis and aims

The shaping of the adaptive immune repertoire diversity and the fine balance existing between immune protection towards pathogens and the onset of self-reactivity have fascinated immunologists since the time of Paul Ehrlich at the beginning of the 20th century. This explains why several groups have focused their studies on the mechanisms that control and silence autoreactive B cells during the B cell development both under physiological conditions and most importantly during autoimmunity.

The importance of B cell activation and autoantibodies in SS and RA is not limited to the clinical diagnosis of these autoimmune diseases. For instance, antibodies from SS patients have been shown to directly mediate exocrine dysfunction most likely by inhibiting muscarinic M3 receptor activity. Similarly, antibodies against citrullinated proteins (ACPA) have been implicated in the pathogenesis of RA.

However, i) the mechanisms regulating breach of humoral B cell tolerance and the development/maintenance of autoimmunity in the periphery and in ELS; ii) the nature and fine specificities of the (auto)antigens driving B cell autoimmunity and iii) the exact pathogenic role of autoantibodies in RA and SS have not been clarified. The possibility to tackle the above issues would provide a better understanding of the pathogenesis of autoimmune diseases, particularly regarding those characterised by functional B cell activation within a target organ, such as the RA synovium and the SS salivary glands.

One of the limitations in order to address such important aspects is the technical challenge of investigating the fine specificity of autoreactive B cells isolated from the inflammatory milieu. In addition, the possibility to analyse B cell autoreactivity from

circulating and/or lesional B cells of autoimmune patients at single cell level would allow a better understanding of the frequency and Ig gene repertoire underlying the development of autoimmunity.

In order to overcome such limitations, in this PhD thesis I have applied the breakthrough strategy developed some years ago in the Nussenzweig's lab [34] which allows cloning and expression of recombinant monoclonal antibodies from single human B cells. This method has the significant advantage of combining the characterization of the Ig gene usage among specific B cell subpopulations from patients with autoimmune diseases and healthy individuals linked with the expression of recombinant antibodies bearing the identical antigenic specificity of the parental B cell in vivo.

As such, the main aim of my project was to develop and validate such technique in my laboratory and characterize the autoreactive B cell response and the impairment in tolerance checkpoints i) in the peripheral blood of patients with SS and ii) from lesional B cells isolated from RA synovial tissues characterised by functional ELS.

Results of my work are organised in this thesis as follows:

- Chapter 4 is focused on the characterization of the V gene repertoire and the frequency of self- and poly-reactive circulating naïve B cells from patients with SS in order to investigate the possible presence of abnormalities of B cell tolerance checkpoints in SS patients.

- Chapter 5 describes the characterization of the V gene repertoire and somatic mutation rate of circulating IgD-CD27+ memory switched and IgD+CD27+ memory unswitched single B cells from patients with SS. Furthermore, characterization of their polyreactivity profile is included.

- Chapter 6 is centred on the generation and characterization of recombinant monoclonal antibodies isolated from CD19+ B cells infiltrating the synovial tissue of ELS+ RA patients. Analysis of the V gene repertoire, VH gene somatic mutation rate and clonal diversification of the synovial B cell clones forms the main part of this chapter together with the characterization of their immunoreactivity towards citrullinated antigens.

In the short-term, this project allowed me to generate a large number of recombinant antibodies from SS and RA patients and characterise their autoreactivity profile against known autoantigens.

In the long-term, my plan is to use such unique tools in order to i) identify dominant antigenic epitopes (self and non-self) recognised among different lesional B clones from different patients with RA and SS and ii) test the direct pathogenic role of in situ-generated autoantibodies in contributing to autoimmunity and chronic inflammation.

Chapter 3 | Patients and Methods

3 | Patients and Methods

3.1 Sjögren's syndrome patients and controls.

Peripheral blood was obtained using venepuncture from 12 patients with a diagnosis of SS (11 primary, 1 secondary to RA) according to the American-European Consensus Group classification criteria after informed consent (LREC 05/Q0707/1) [48]. The main demographic and clinical characteristics of the RA patients analysed are reported in **Table 3.1**. Naïve B cells generated from one previously reported healthy donor (HD-JH [177] and two additional HD - HD2 and HD3) were used as controls.

3.2 Rheumatoid arthritis patients.

Synovial tissue was obtained from 4 patients with a diagnosis of RA according to the American College of Rheumatology criteria [100]. Synovial tissue was collected after informed consent (LREC 05/Q0703/198) by total joint replacement. The demographics and clinical features of the RA patients are summarized in **Table 3.1**.

3.3 Human Peripheral blood mononuclear cells (PBMCs) preparation.

PBMCs were obtained from heparinized blood by centrifugation on Ficoll-Paque gradients. Blood was diluted with 1X PBS, supplemented with 2.5 mM EDTA and loaded over a Ficoll-Paque 1077. Density gradient centrifugation was performed at 800xg at room temperature for 25 min without break to separate mononuclear cells. The mononuclear cell layer appears as a cloudy ring at the PBS/Ficoll-Paque interface. Finally, the mononuclear cells were harvested from the interface and transferred to a

new tube and washed twice with PBS (500xg and 400xg with break, respectively). PBMCs viability was determined by Trypan blue exclusion test.

SS patients	*SS1*	*SS2*	*SS3*	*SS4*	*SS5*	*SS6* (*)	*SS8*	*SS9*	*SS10*	*SS11*	*SS12*	*SS13*
Age	64	64	59	54	39	59	68	64	48	62	41	56
Gender	F	F	F	F	F	F	F	F	F	M	F	F
Age SS onset	52	50	43	44	32	43	66	62	43	53	26	46
Disease duration (Years)	12	14	16	10	7	16	2	2	5	9	14	10
Diagnosis	pSS	pSS	pSS	pSS	pSS	pSS	pSS	pSS	sSS/RA	pSS	pSS	pSS
Anti-Ro/SSA	+	+	+	+	+	+	-	-	+	+	+	+
Anti-La/SSB	+	-	-	+	-	-	-	-	-	+	+	+
Treatment	HCQ	HCQ	HCQ	HCQ	N	N	N	HCQ	HCQ MTX	HCQ	HCQ PDN 5mg	HCQ PDN 5mg

RA patients	*RA1-3 (**)*	*RA2*	*RA4*	*RA5*
Age	66	75	39	63
Gender	F	F	F	F
Age of onset	61	na	35	59
RF	+	-	+	na
Anti-CCP	+	na	na	+
Treatment	MTX, SSZ, anti-TNF	MTX, anti-TNF, RTX	MTX, anti-TNF	MTX, RTX

Table 3.1 Demographic and clinical features of the SS and RA patients analysed in this study.

pSS=primary Sjögren's syndrome; sSS=secondary Sjögren's syndrome; RA=rheumatoid arthritis; na=not available

HCQ= Hydroxychloroquine; MTX= Methotrexate; PDN= prednisolone; RTX=Rituximab; SSZ=Sulphasalazine; N=no treatment

(*) Patient SS7 (not shown in the table) is the same as patient SS13 but analysed at different time points

(**) Patient RA1 is the same as patient RA3 but two joints replacement (left and right knee, respectively)

3.4 Preparation of mononuclear cells from synovial tissue.

Mononuclear cells were isolated from synovial tissue specimens obtained from hip or knee joint replacement surgery. The extracellular matrix in animal tissues is made by collagens and other extracellular matrix proteins such as glycoproteins and proteoglycans. To isolate single cells without disrupting the cellular structure a combination of proteolytic enzymes is necessary to dissociate the tissue. Therefore, the synovial tissue was cut into small pieces and enzymatically digested in 1.5 ml RPMI (supplemented with 2% FBS) with 37 µl collagenase D (100 mg/ml, Roche) and 2 µl DNase I (10 mg/ml) at 37 °C for 1 hour under shaking in a water-bath with tiny (~1-1.5 cm long) magnetic stirrers. After the first digestion, the sample was incubated in 1.5 ml RPMI (supplemented with 2% FBS) with 37 µl collagenase/dispase mix solution (100 mg/ml, Roche) and 2 µl DNase I (10 mg/ml) at 37 °C for 30 min under shaking in the water-bath. After the second incubation, 15 µl of 0.5 M EDTA were added to stop the reaction. Collagenase is an enzyme which is used for the dissociation of tissues since it breaks down the native collagen that holds tissues together. In particular, collagenase D is used to preserve functionality and integrity of cell-surface proteins. Dispase is a protease which is able to cleave fibronectin, collagen IV, and to a lesser extent collagen I. It is used for gentle dissociation with minimal cell damage. Both enzyme activities are inhibited by the addition of EDTA.

The sample was then filtered through 40 µm cell strainer (Sigma) to remove the remaining pieces of tissue and centrifuged at 1200 rpm for 10 min. The cells were resuspended in complete tissue culture media. Cells viability was determined by

Trypan blue exclusion test. The isolated cells were put in culture for 4 days in the presence of BAFF (100 ng/ml) in the culture media before starting the isolation of single CD19+ B cells by FACS sorting (see paragraph 3.5). A schematic summary of the procedure is provided in **Figure 3.1**.

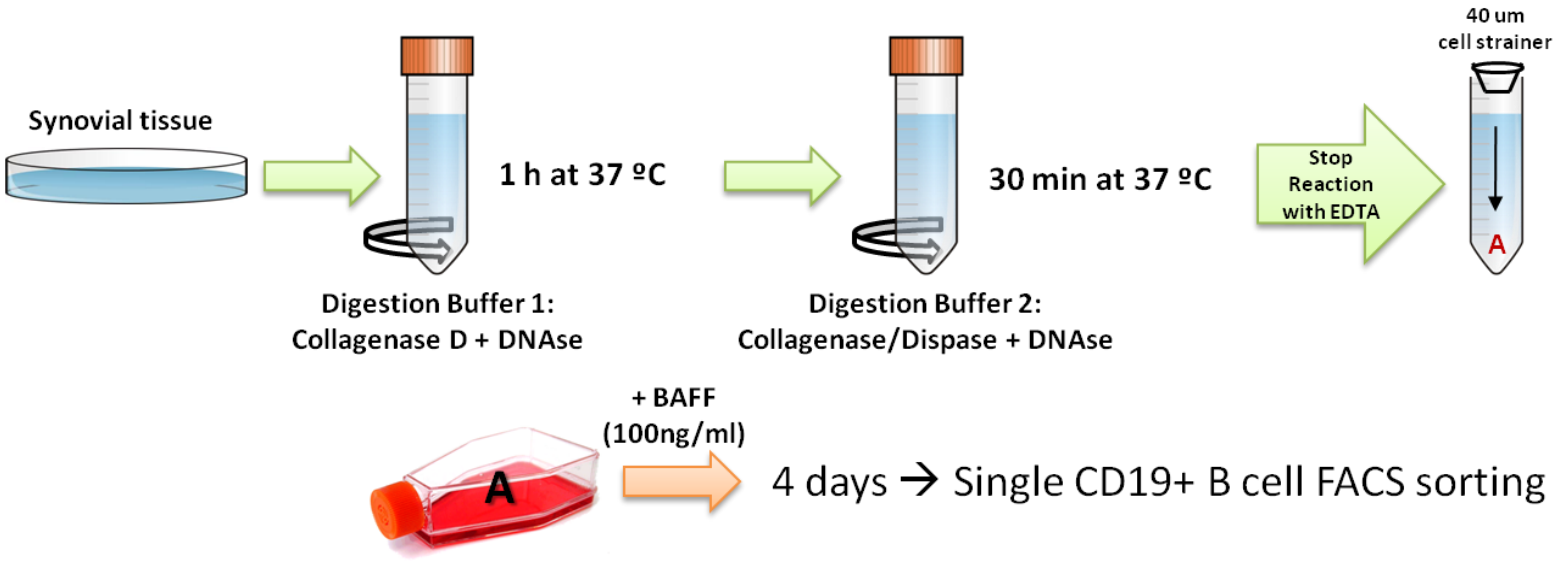

Figure 3.1 Preparation of mononuclear cells from synovial tissue.

Schematic representation of synovial tissue digestion used to isolate mononuclear cells in order to sort single CD19+ B cells by FACS.

3.5 Fluorescence-activated cell sorting (FACS).

Flow cytometry is a technique to measure optical and fluorescence characteristics of single cells. Inside a flow cytometer, cells in suspension are drawn into a stream which allows the cells to pass individually through a specific point where a beam of light (laser light) of single wavelength crosses the cells. Laser light is scattered in all directions. Light which scatters in line with the laser beam is called forward scatter (FSC); light that scatters perpendicular to the laser beam is called side scatter (SSC). FSC and SSC are related to physical properties of a cell which are size and internal complexity or granularity, respectively. When cells are stained with fluorochrome-conjugated antibodies and passed through a laser beam, the fluorescent dyes can absorb energy and be induced to an excited electronic state. In returning to their normal level, the dyes release energy, most of which is emitted as light known as fluorescence emission. The scattered and fluorescent light is detected by photomultiplier tubes and analyzed by a computer. The data generated are plotted in a single dimension (histogram) or in two dimensions (dot plots). Fluorescence-activated cell sorting (FACS) is a specific type of flow cytometry since it used for sorting a mixture of cells into two or more tubes or directly into 96/384-well plates, one cell at the time, based on the specific light scattering and fluorescent characteristics of each cell (**Figure 3.2**). Briefly, during the sorting the stream of cells is broken into individually droplets. When a particle is recognized based on predefined sorting criteria, an electrical charge is placed to the stream just as the droplet containing that

particle break off the stream. The charged droplets then fall through two charged plates that divert droplets into containers depending on the droplet charge.

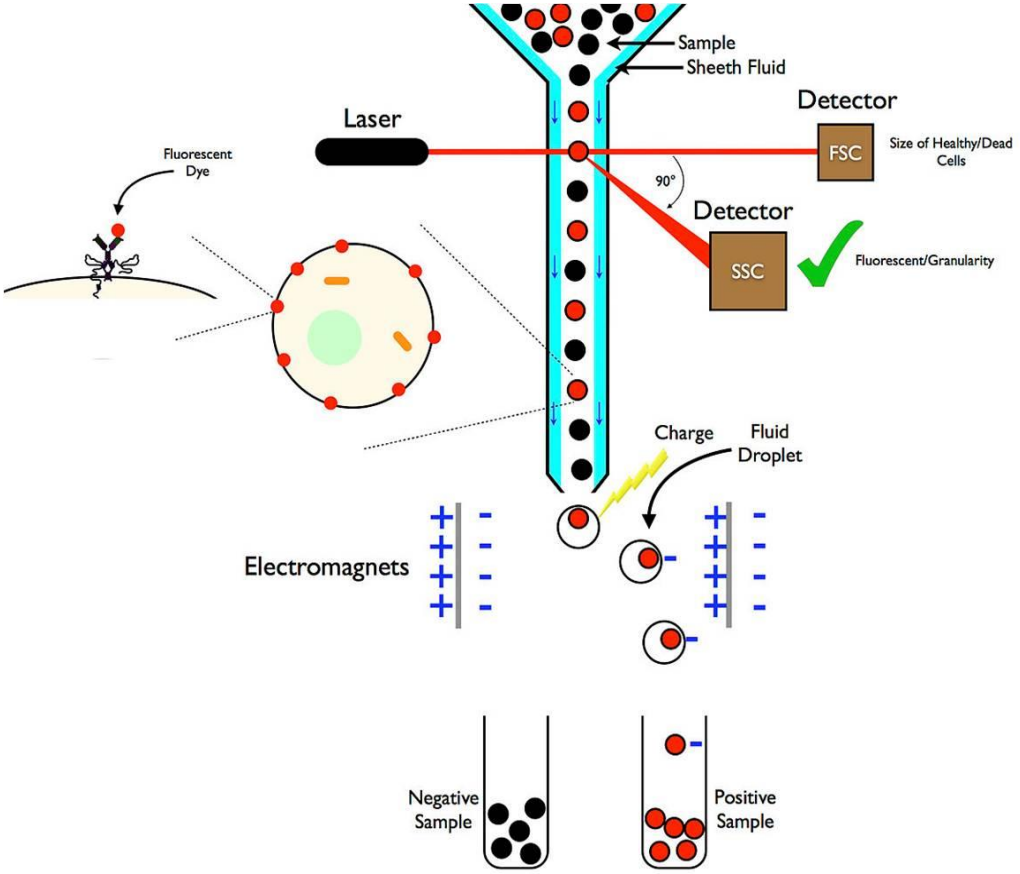

Figure 3.2 Schematic explanation of fluorescent-activated cell sorting (FACS).

3.6 Phenotypic characterization of B cells by fluorescence activated cell sorting (FACS).

Immunofluorescence labeling for flow cytometry was performed by staining the purified mononuclear cells on ice with PerCP-Cy5.5 anti-human CD19, APC anti-human CD27, PE anti-human IgD, and FITC anti-human CD3 in order to differentiate CD3-CD19+CD27-IgD+ naïve B cells, CD3-CD19+CD27+IgD+ unswitched memory B cells and CD3-CD19+CD27+IgD- switched memory B cells, as previously described [82]. Mononuclear cells isolated from synovial tissues were stained only with PerCP-Cy5.5 anti-human CD19 and FITC anti-human CD3, in order to isolate single CD19+ B cells. Mononuclear cells were aliquoted into an Eppendorf tube and centrifuged at 4300 rpm at 4 °C for 4 min. The cell pellet was resuspended in 100 μl of FACS buffer staining solution containing all the antibodies at the appropriate final concentration (**Table 3.2**). Incubation with antibodies was performed in the dark at 4°C for 30 min. The cells were washed with 1 ml FACS buffer and resuspended in FACS buffer at a final concentration of 10^7cell/ml. Finally, a blue-fluorescent dye (DAPI) which stains the nuclei was added to exclude dead cells. Flow cytometric analysis was performed with a FACSAria flow cytometer (Becton Dickinson); 50,000 events were collected for each analysis. Single staining was used for setting up the flow cytometer and to perform fluorescence compensation. It was done using Becton Dickinson compensation beads, according to the manufacturer's instruction. Further controls were added and they were the fluorescence minus one (FMO) controls to set appropriately the cell population gates which is an important step especially for the single cell sorting.

Antibody	Clone	Conjugation	Dilution	Source
CD19	SJ25C1	PerCP-Cy5.5	1/10	BD Biosciences
IgD	IA6-2	PE	1/10	BD Biosciences
CD27	O323	APC	1/100	eBioscience
CD3	HIT3a	FITC	1/200	eBioscience

Table 3.2 List of antibodies used for FACS.

3.7 Isolation of single human B cells by fluorescence activated cell sorting.

Single cells were sorted on a FACSAria flow cytometer directly into 96-well plates (Eppendorf) containing 4 μl/well of ice-cold 0.5X PBS, 100 mM DTT (Invitrogen), 40 U/μl RNasin Ribonuclease Inhibitor (Promega) as previously described [34]. Plates were sealed with adhesive PCR foil (4titude) and immediately frozen on dry ice before storage at -80 °C.

3.8 Single cell RT-PCR and immunoglobulin V gene amplification.

cDNA was synthesized in a total volume of 14.5 μl per well in the original 96-well sorting plate as previously described [34]. Total RNA from single cells was reverse transcribed in nuclease-free water (Qiagen) using 300 ng/μl random hexamer primers (Roche), 25 mM each nucleotide dNTP-mix (Invitrogen), 100 mM DTT (Invitrogen), 10% NP-40 (Sigma), 40 U/μl RNasin (Promega), and 50 U Superscript III reverse transcriptase (Invitrogen).

Reverse transcription (RT) reaction was performed at 42 °C for 5 min, 25°C for 10 min, 50 °C for 60 min and 94 °C for 5 min. cDNA was stored at -20 °C. For each cell IgH and corresponding IgL chain (Igκ and Igλ) gene transcripts were amplified independently by nested PCR starting from 2-3 μl of cDNA as template. The nested PCR is a modification of the common PCR in which two sets of primers are used in two consecutive rounds of PCR. The second set of primers bind the first PCR product internally ensuring that

the product from the second PCR has little contamination, thus increasing the sensitivity and specificity of the PCR.

All PCR reactions were performed in 96-well plates in a total volume of 40 μl per well containing 50 mM each primer, 25 mM each nucleotide dNTP-mix (Invitrogen) and 1.2 U HotStar Taq DNA polymerase (Qiagen) (**Table 3.3 and 3.4**) [34]. The HotStar Taq DNA polymerase has an error rate of $2x10^{-5}$ / nucleotide and cycle and it was chosen instead of high fidelity enzymes which introduce less error because the HotStar Taq DNA polymerase has a high amplification efficiency for low copy templates when starting from single cells [34]. All the primers used in this study were the same as previously published [34] with the exception in the use of a novel Cμ internal primer (GGGAATTCTCACAGGAGACGA) in the second round of the nested PCR. All nested PCR reactions with family-specific primers were performed with 3 μl of unpurified first PCR product. Family-specific primers were selected based on the Ig gene sequence analysis (see paragraph 2.10). Each round of PCR was performed for 50 cycles at 94 °C for 30 s, 58 °C (IgH/Igκ) or 60 °C (Igλ) for 30 s, 72 °C for 55 s (1^{st} PCR) or 45 s (2^{nd} PCR). All PCR products were analyzed on 2% agarose gels (~ 500 base pairs (bp) for IgH chain; ~ 400 bp for IgL chains). A schematic representation of the strategy for single B cell sorting and Ig VH and VL genes amplification is reported in **Figure 3.3**.

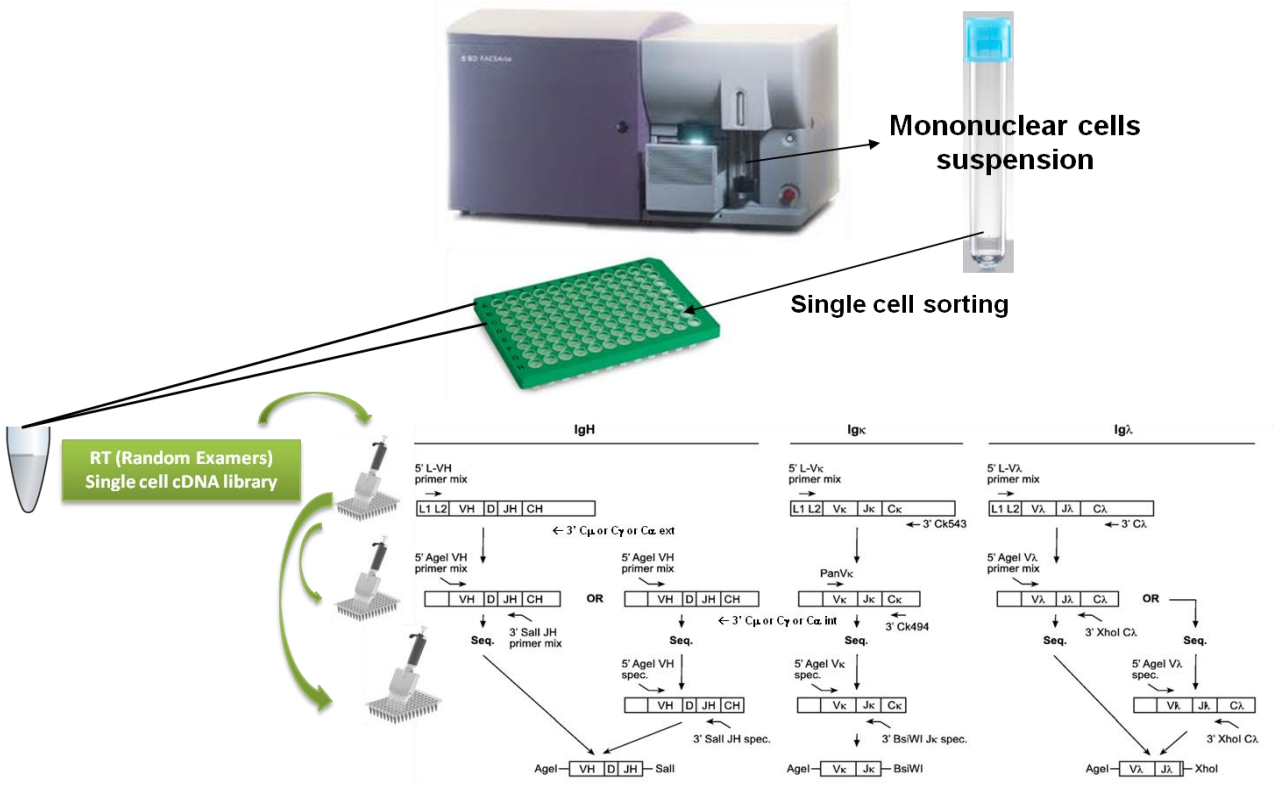

Figure 3.3 FACS single cell sorting and immunoglobulin V gene amplification.

Single cells were sorted on a FACSAria directly into a 96-well plate. cDNA was synthesized in the original sorting plate and used to amplify for each cell IgH, Igκ and Igλ independently by nested PCR.

3.8.1 DNA electrophoresis on agarose gel.

Agarose (Fischer Scientific) gels with a concentration of 2.0% in 1X TBE buffer were used to separate PCR products. DNA fragments were visualized by staining the gels with ethidium bromide (Sigma) in a soaking solution prepared with 25 μl of ethidium bromide in 0.5 L distilled water. 100 bp marker (NEB) ranging from 100-1,517 bp was used as molecular weight standard.

3.9 Ig gene sequence analysis.

Aliquots of VH, Vκ and Vλ chains second PCR products were sequenced with the respective reverse primer (Beckman Coulter Genomics) and the sequences were analyzed by IgBlast (http://www.ncbi.nlm.nih.gov/igblast/) to identify germline V(D)J gene segments with highest homology. IgH complementary determining region CDR3 length and the number of positively (Histidine (H), Arginine (R), Lysine (K)) and negatively charged (Aspartate (D), Glutamate (E)) amino acids were determined as previously described [33, 34]. CDR3 length was determined as indicated in IgBlast by counting the amino acid residues following framework region FR3 up to the conserved tryptophan-glycine motif in all JH segments or up to the conserved phenylalanine-glycine motif in JL segments. The numbers of V gene somatic mutations (replacement and silent mutations) were counted manually for FR1, FR2, FR3 and CDR1 and CDR2. Ratios of replacement to silent mutations in FR1-FR3 and CDR1 and CDR2 were calculated for each IGH, IGK and IGL chain gene and expressed as total number of sequences analysed for each IGH, IGK and IGL chain gene.

The V gene somatic mutations for the clones isolated from the RA synovium was performed using IMGT/V-QUEST search page (http://imgt.org/IMGT_vquest). IMGT/V-QUEST is a tool which is used to analyse the Ig nucleotide sequences [178]. Similar to IgBlast, IMGT/V-QUEST identifies the V, D and J genes and alleles in rearranged V-J and V-D-J sequences by alignment with germline Ig gene and allele sequences of the IMGT directory. It defines the FR regions and CDR regions and provides a detailed characterization of the query sequence. The algorithm used to identify the closest V, D,

and J genes and alleles is based on global pairwise alignment followed by a similarity evaluation. In particular, I used the nucleotide mutation option available on the IMGT/V-QUEST website which characterises the silent versus non-silent mutation in each FR region and CDR region in order to determine the R/S ratio.

B cell population	IgH Reverse primer	Igκ Reverse primer	Igλ Reverse primer
CD3-CD19+CD27-IgD+ naïve	1st PCR: Cμ CH1 ext 2nd PCR: Cμ CH1 int	1st PCR: Cκ 543 2nd PCR: Cκ 494	1st PCR: Cλ 2nd PCR: XhoI Cλ
CD3-CD19+CD27+IgD-memory switched	1st PCR: Cγ CH1 ext 2nd PCR: Cγ IgG int 1st PCR: Cα CH1 ext 2nd PCR: Cα CH1-2 int	1st PCR: Cκ 543 2nd PCR: Cκ 494	1st PCR: Cλ 2nd PCR: XhoI Cλ
CD3-CD19+CD27+IgD+ memory unswitched	1st PCR: Cμ CH1 ext 2nd PCR: Cμ CH1 int	1st PCR: Cκ 543 2nd PCR: Cκ 494	1st PCR: Cλ 2nd PCR: XhoI Cλ
CD3-CD19+	2nd PCR: Cμ CH1 int 2nd PCR: Cμ CH1 int 1st PCR: Cγ CH1 ext 2nd PCR: Cγ IgG int 1st PCR: Cα CH1 ext 2nd PCR: Cα CH1-2 int	1st PCR: Cκ 543 2nd PCR: Cκ 494	1st PCR: Cλ 2nd PCR: XhoI Cλ

Table 3.3 Reverse primers used to amplify IgH and IgL chain gene transcripts.

IgH chain gene transcripts from SS naïve, memory switched and unswitched B cells were amplified using primers that bind the Cμ, Cγ or Cα constant region as indicated in the table for each population. cDNA from CD3-CD19+ B cells isolated from synovial tissue was amplified using primers that bind the Cμ, Cγ or Cα constant region in three independent nested PCR. Igκ and Igλ chain gene transcripts were amplified independently using same reverse primers for all the B cell populations.

Primer	Sequence
1st PCR HC	Forward 5' to 3'
5' L-VH 1	ACAGGTGCCCACTCCCAGGTGCAG
5' L-VH 3	AAGGTGTCCAGTGTGARGTGCAG
5' L-VH 4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
5' L-VH 5	CAAGGAGTCTGTTCCGAGGTGCAG
	Reverse 3' to 5'
3' Cμ CH1 ext	GGAAGGAAGTCCTGTGCGAGGC
3' Cγ CH1 ext	GGAAGGTGTGCACGCCGCTGGTC
3' Cα CH1 ext	TGGGAAGTTTCTGGCGGTCACG
2nd PCR HC	Forward 5' to 3'
5' AgeI VH1	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGGTGCAG
5' AgeI VH1/5	CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG
5' AgeI VH 1-18	CTGCAACCGGTGTACATTCCCAGGTTCAGCTGGTGCAG
5' AgeI VH 1-24	CTGCAACCGGTGTACATTCCCAGGTCCAGCTGGTACAG
5' AgeI VH3	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG
5' AgeI VH3-23	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG
5' AgeI VH3-33	CTGCAACCGGTGTACATTCTCAGGTGCAGCTGGTGGAG
5' AgeI VH 3-9	CTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG
5' AgeI VH4	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG
5' AgeI VH 4-34	CTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG
5' AgeI VH4-39	CTGCAACCGGTGTACATTCCCAGCTGCAGCTGCAGGAG
5' AgeI VH 6-1	CTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG
	Reverse 3' to 5'
3' Cμ CH1 int	GGGAATTCTCACAGGAGACGA
3' Cγ IgG int	GTTCGGGGAAGTAGTCCTTGAC
3' Cα CH1-2 int	GTCCGCTTTCGCTCCAGGTCACACT

Table 3.4 Complete list of primers used in this study.

The table lists the primers used to amplify the Ig heavy chain.

1st PCR λ LC	Forward 5' to 3'
5' L Vλ 1	GGTCCTGGGCCCAGTCTGTGCTG
5' L Vλ 2	GGTCCTGGGCCCAGTCTGCCCTG
5' L Vλ 3	GCTCTGTGACCTCCTATGAGCTG
5' L Vλ 4/5	GGTCTCTCTCSCAGCYTGTGCTG
5' L Vλ 6	GTTCTTGGGCCAATTTTATGCTG
5' L Vλ 7	GGTCCAATTCYCAGGCTGTGGTG
5' L Vλ 8	GAGTGGATTCTCAGACTGTGGTG
	Reverse 3' to 5'
3' Cλ	CACCAGTGTGGCCTTGTTGGCTTG
2nd PCR l LC	Forward 5' to 3'
5' AgeI Vλ 1	CTGCTACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG
5' AgeI Vλ 2	CTGCTACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG
5' AgeI Vλ 3	CTGCTACCGGTTCTGTGACCTCCTATGAGCTGACWCAG
5' AgeI Vλ 4/5	CTGCTACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA
5' AgeI Vλ 6	CTGCTACCGGTTCTTGGGCCAATTTTATGCTGACTCAG
5' AgeI Vλ 7/8	CTGCTACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG
	Reverse 3' to 5'
3' XhoI Cλ	CTCCTCACTCGAGGGYGGGAACAGAGTG
1st PCR κ LC	Forward 5' to 3'
5' L Vκ 1/2	ATGAGGSTCCCYGCTCAGCTGCTGG
5' L Vκ 3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
5' L Vκ 4	ATTTCTCTGTTGCTCTGGATCTCTG
	Reverse 3' to 5'
3' Cκ 543	GTTTCTCGTAGTCTGCTTTGCTCA
2nd PCR k LC	Forward 5' to 3'
5' Pan Vκ	ATGACCCAGWCTCCABYCWCCCTG
	Reverse 3' to 5'
3' Cκ 494	GTGCTGTCCTTGCTGTCCTGCT
Specific k LC	Forward 5' to 3'
5' AgeI Vκ 1-5	CTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC
5' AgeI Vκ 1-9	TTGTGCTGCAACCGGTGTACATTCAGACATCCAGTTGACCCAGTCT
5' AgeI Vκ 1D-43	CTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC
5' AgeI Vκ 2-24	CTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC
5' AgeI Vκ 2-28	CTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC
5' AgeI Vκ 2-30	CTGCAACCGGTGTACATGGGGATGTTGTGATGACTCAGTC
5' Age Vκ 3-11	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACACAGTC
5' Age Vκ 3-15	CTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGTC
5' Age Vκ 3-20	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACGCAGTCT
5' Age Vκ 4-1	CTGCAACCGGTGTACATTCGGACATCGTGATGACCCAGTC
	Reverse 3' to 5'
3' BsiWI Jκ 1/4	GCCACCGTACGTTTGATYTCCACCTTGGTC
3' BsiWI Jκ 2	GCCACCGTACGTTTGATCTCCAGCTTGGTC
3' BsiWI Jκ 3	GCCACCGTACGTTTGATATCCACTTTGGTC
3' BsiWI Jκ 5	GCCACCGTACGTTTAATCTCCAGTCGTGTC

Table 3.4 Complete list of primers used in this study. The table lists the primers used to amplify the Ig light chains (κc and λc).

3.10 Expression vector cloning.

Before cloning, all PCR products were purified with NucleoSpin Extract II (Macherey-Nagel) according to the manufacturer's instruction and the digests were carried out with the respective restriction enzymes HF AgeI (20 units/μl), HF SalI (20 units/μl), BsiWI (10 units/μl) and HF XhoI (20 units/μl) (all from NEB). The digestion was performed using one unit of enzyme per reaction for 2 hour at 37 °C (AgeI, SalI and XhoI) and a further incubation at 55 °C for 2 hours for BsiWI (kappa chain digestion) in 40 μl reaction volume. Digested PCR products were purified with NucleoSpin Extract II and ligated using the T4 DNA Ligase (NEB) into human IgG1, IgK or IgL expression vector (**Figure 3.4**) for 1 hour at room temperature in 10 μl reaction volume. The vectors were kindly provided by Prof Hedda Wardemann (Berlin, Germany).

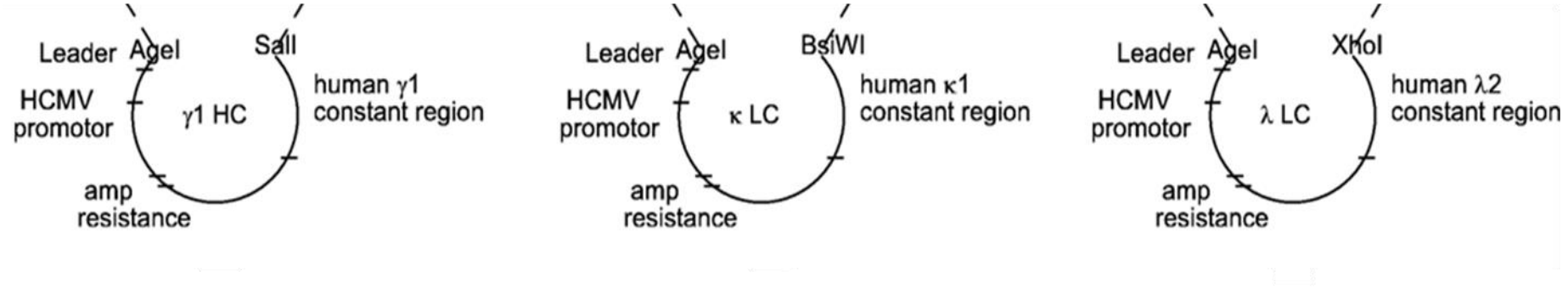

Figure 3.4 Schematic expression vector maps [34].

Eukaryotic expression vectors contain a murine Ig gene leader peptide sequence (GenBank accession no. DQ407610) and a multiple cloning site upstream of the human Igγ1, Igκ or Igλ constant regions. Relevant restriction sites for each vector are indicated. Transcription is under the influence of the human cytomegalovirus (HCMV) promoter and bacterial clones containing the vectors can be selected based on their resistance to ampicillin.

Competent E. coli DH10B bacteria (NEB) (5 μl per reaction) were thawed on ice and 3 μl of the ligation product were subsequently added. Cells and DNA were incubated on ice for 30 min. This step was followed by an Heat Shock at 42 °C for 20 sec. Cells and DNA were left to incubate at 37 °C for 30 min with shaking at 180 rpm. Cells and DNA were finally plated into selection LB plates containing 100 μg/ml ampicillin and left to incubate at 37 °C over night. Colonies were screened by PCR using 5' Absense (GCTTCGTTAGAACGCGGCTAC) as forward primer and 3' Cγ IgG internal, 3' Cκ494 or 3' Cλ as reverse primer, respectively. PCR products (analyzed on 2% agarose gels) of the expected size (650 bp for Igγ1, 700 bp for Igκ and 590 bp for Igλ) were sequenced to confirm identity with the original PCR products. Plasmid DNA was isolated from 4 ml of bacteria cultures grown for 16 hours at 37 °C on a shaker (200 rpm) in Terrific Broth (TB) medium containing 100 μg/ml Ampicillin, using NucleoSpin Plasmid (Macherey-Nagel). Before purification bacteria cultures were centrifuged for 5 min at 6000 rpm in a microcentrifuge. Supernatant was discarded and the plasmid pellet used to start the purification procedure, according to the manufacturer's instruction. After purification plasmid concentration was measured with Nanodrop 2000c (ThermoScientific).

3.11 Recombinant antibody production.

To express the antibodies *in vitro*, HEK 293T cells cultured in 150 mm or 6-well plates (Falcon, BD) were co-transfected with plasmids encoding the IgH and IgL chains originally amplified from the same B cell. Transient transfection of exponentially growing 293T cells was performed by Polyethylenimine (PEI) at 60-70% cell confluency. PEI is a cationic polymer able to condense the DNA into positively charged particles

which are brought into the cells via endocytosis. Once inside the cell, the vesicle releases the polymer-DNA complex into the cytoplasm. If the complex unpacks then the DNA is able to diffuse into the nucleus.

On transfection day complete medium was washed off and replaced with "Nutridoma" medium before the addition of the transfection solution. Transfection solution was prepared by adding each heavy and light chain plasmid at the same concentration (15 μg for 150 mm plate and 0.81 μg for 6-well plate), 150 mM NaCl solution, and PEI at a 3:1 ratio (weight/weight) with total vector DNA (3 μg of PEI per μg DNA). The solution was mixed thoroughly and incubated at room temperature for 10 min and then added to the cells. The cells secreted the antibodies into the tissue culture supernatant which was collected at day 3 and 5. Culture supernatant was cleared from cell debris by centrifugation at 2000 rpm for 10 min and stored at 4 °C with 0.05% NaN_3. Recombinant antibody concentrations were determined by ELISA (see paragraph 3.13) before purification with Protein G beads (GE Healthcare).

3.12 Purification of monoclonal antibodies using Protein G Beads.

Protein G binds to the Fc region of IgG from a variety of mammalian species. Protein G Sepharose 4 Fast Flow (GE Healthcare) is used to isolate and purify classes, subclasses and fragments of immunoglobulins from any biological fluid or cell culture medium. Cell culture supernatant was incubated with 1 μl/ml of Protein G beads for at least 14 hours at 4 °C under rotation. Supernatant was removed after centrifugation at 2000 rpm for 10 min and the beads were transferred to a chromatography spin column

(BioRad) equilibrated with PBS. After two rounds of washing with 1 ml PBS, antibodies were eluted in 3–4 fractions (200 µl each) with 0.1 M glycine (pH 3.0). Eluates were collected in tubes containing 20 µl 1 M Tris-HCl (pH 9.0) with 0.5% NaN_3. Recombinant antibody concentrations were determined by ELISA (see paragraph 3.13).

3.13 Human IgG ELISA test.

Recombinant antibody concentrations were determined by ELISA using 1 µg/ml and 3 µg/ml human serum IgG1 (Sigma) diluted in PBS as standards. ELISA plates (Thermo Scientific) were coated with 50 µl/well goat anti-human IgG (Fcγ specific; Jackson) at a concentration of 2 µg/ml in 1X PBS. Plates were washed with deionized water before incubation for 1 hour with 200 µl/well 2mM EDTA and 0.05% Tween 20 in 1X PBS and washed again. Standard and samples were transferred into the ELISA plate and incubated for 1 hour. Unbound antibodies were removed by washing before incubation for 1 hour with 50 µl/well of horseradish peroxidise (HRP) coupled goat anti-human IgG (Jackson) at a concentration of 0.8 µg/ml in 1X PBS, 2 mM EDTA, 0.05% Tween 20. Assays were developed using TMB Substrate Reagent Set (BD OptEIA). Optical densities (OD) were measured at 450 nm. All steps were performed at room temperature.

3.14 Characterization of polyreactivity and self-reactivity by ELISA.

To test the reactivity against different allo- and auto-antigens, supernatants were tested for polyreactivity against double and single-stranded DNA (dsDNA and ssDNA), lipopolysaccharide (LPS) and insulin by ELISA, as previously reported [34]. Antibodies

that reacted against at least two structurally diverse self- and non-self-antigens were defined as polyreactive [33, 34]. Internal controls for polyreactivity were added on each plate consisting of the recombinant monoclonal antibodies mGO53 (negative), JB40 (low polyreactive), and ED38 (highly polyreactive), as previously reported [34]. ELISA plates were coated with 50 μl/well of individual antigens at a concentration of 10 μg/ml for dsDNA (Invitrogen), ssDNA and LPS (Sigma), or 5 μg/ml for insulin (Sigma) in 1X PBS. Each plate was washed with deionized water before incubation for 1 hour with 200 μl/well 2mM EDTA and 0.05% Tween 20 in 1X PBS and washed again. Samples and controls were used at three consecutive 1:4 dilutions in 1X PBS starting from 1 μg/ml and were then transferred into the ELISA plate and incubated for 1 hour. Unbound antibodies were removed by washing before incubation for 1 hour with 50 μl/well of HRP coupled goat anti-human IgG at a concentration of 0.8 μg/ml in 1X PBS, 2 mM EDTA, 0.05% Tween 20. Assays were developed using TMB Substrate Reagent Set (BD OptEIA). Optical densities (OD) were measured at 450 nm. All steps were performed at room temperature.

3.14.1 Total ENA, anti-SSA/Ro and anti-SSB/La ELISA.

Protein G purified antibodies were also tested for reactivity against extractable nuclear antigens (ENAs) as well as anti-Ro/SSA and anti-La/SSB using commercially available ELISA kits commonly used in diagnostic labs (DIASTAT ENA Single Well Screen and anti-Ro/La ELISA plates, Euro Diagnostica). In the ENA ELISA each well is coated with a mix of six purified antigens (Sm, RNP, Ro/SSA, La/SSB, Scl-70 and Jo-1). All ELISA tests were performed according to the manufacturer's instruction. Briefly, controls and samples

were added to the antigen(s) coated plate and incubated for 1 hour at room temperature at a concentration of 10 μg/ml. Each plate was washed and incubated with 100 μl of Conjugate (enzyme labelled antibodies to human IgG) for 30 min. After further washing the assay was developed using the Substrate solution provided by the kit. OD were measured at 550 nm. Besides the internal controls provided by the ELISA kit, a further control used for the ENA and anti-Ro/SSA ELISA was the recombinant monoclonal antibody SLE175-128 that shows specificity for Ro52 [177].

3.15 Indirect Immunofluorescence Assay (IIF) using Hep-2 cells.

Self-reactivity against anti-nuclear antigens (ANA) was tested by indirect-immunofluorescence assay (IIF) using human epithelial cells (Hep-2) coated slides as substrate (ByoSystems), as previously reported [34]. ANA Hep-2 is used normally for determination of antibodies to nuclear and cytoplasmic antigens in clinical diagnostic. Several patterns can be distinguished after fluorescence exposure based on the nuclear morphology and mitosis phase of the Hep-2 cell. The main nuclear patterns are classified as follows: homogeneous, peripheral (rim), speckled (coarse or fine), nucleolar, and centromere. In this study, the result was given only as negative, nuclear, cytoplasmic and mixed nuclear/cytoplasmic staining patterns. Hep-2 slides were incubated with 20 μl of protein G purified antibodies at 10 μg/ml for 1 hour at room temperature in a moist chamber. After washing with 1X PBS the slides were incubated for 30 min with Alexa Fluor 488-conjugated goat anti-human IgG in the dark (Invitrogen, dilution 1/200). Controls staining with PBS as negative control and ANA

positive control sera were added in all experiments. Hep-2 staining patterns were visualised using an Olympus BX60 microscope and digital images acquired using identical exposure times throughout (1000 msec). ANA were scored independently by 3 trained observers and considered positive in case of concordance by at least 2 observers.

3.16 Anti-CCP ELISA test.

Protein G purified antibodies generated from RA synovial B cells were tested towards a synthetic cyclic peptide containing modified arginine residues (CCP2 peptides) using commercially available ELISA kits commonly used in diagnostic labs (Anti-CCP, FCCP600, Axis-Shield). Anti-CCP antibodies, mainly known as anti-citrullinated protein/peptide antibodies (ACPA), are an important diagnostic marker in RA. In the anti-CCP ELISA test, each well is coated with a highly purified synthetic cyclic citrullinated peptide containing modified arginine residues. The anti-CCP ELISA test was performed according to the manufacturer's instruction. Briefly, controls and samples were added to the coated plate and incubated for 1 hour at room temperature at a concentration of 10 μg/ml. After a washing step, the plate was incubated with 100 μl of Conjugate (enzyme labelled polyclonal antibody to human IgG) for 30 min. After further washing the assay was developed using the Substrate solution provided by the kit. OD were measured at 450 nm.

3.17 Histological characterization of lymphocytic aggregates within RA synovial tissue.

Sequential paraffin-embedded 5µm sections of synovial tissue were stained for the markers CD3, CD20 and CD138 following routine H&E staining to classify the lymphocytic infiltration as aggregate or diffuse, as previously reported [179, 180]. Briefly, after de-waxing and re-hydration synovial paraffin sections underwent Ag retrieval with Target retrieval solution (pH 6; DAKO). After incubation with protein block solution (DAKO), staining for the cellular markers CD3 (1:80 dilution; DAKO), CD20 (1:50 dilution, DAKO), and CD138 (1:50 dilution, DAKO) was performed. Primary antibodies were incubated at the appropriate dilution for 1h at room temperature. After 3 washes with TBS, sections were incubated with the EnVision System (DAKO), used according to manufacturer's instructions, for 30 minutes at room temperature. The colour reaction was developed with diaminobenzidine (DAKO). All sections were visualised using an Olympus BX60 microscope.

3.18 Statistical analysis.

Differences in quantitative variables were analyzed by the Mann-Whitney U test when comparing two groups and by the Kruskal-Wallis with Dunn's post-test when comparing multiple groups. $\chi 2$ test with Yates' correction when required or Fisher's exact test when appropriate were used to evaluate associations of qualitative variables in the different groups. All the statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad, San Diego, CA). A p value <0.05 was considered statistically significant. For the ELISAs, the cut-off OD at which all

antibodies were considered reactive was determined for each experiment based on the mean OD plus 2 standard deviations of the control antibodies obtained from HD (naïve B cell antibodies) or other internal controls (mGO53 antibody [34]).

Chapter 4 | Results

Recombinant monoclonal antibodies from single naïve B cells of patients with Sjögren's syndrome

4 | Accumulation of circulating autoreactive naïve B cells reveals defects of early B cell tolerance checkpoints in patients with Sjögren's syndrome.

4.1 Introduction.

As explained in Section 1.2, SS is a chronic inflammatory/autoimmune disease characterised by immune cell infiltration in the salivary and lacrimal glands leading to the classical signs and symptoms of xerostomia (dry mouth) and keratoconjuctivitis (dry eyes) sicca [69]. Together with exocrine dysfunction, the hallmark of SS is the presence of circulating autoantibodies directed against organ- and non-organ-specific autoantigens. Sera of 90% of SS patients are characterised by the presence of antinuclear antibodies (ANA), some of which react against the ribonucleoproteins Ro/SSA (60%) and La/SSB (40%) [88].

Besides the presence of autoantibodies, SS patients are characterised by profound disturbances in the frequency of different B cell subpopulations, both in the peripheral compartment and in the inflamed salivary glands. As explained previously, SS patients show a large predominance of circulating CD27- naïve B cells and a significant reduction of peripheral CD27+ memory B cells, in particular the memory unswitched CD27+IgD+ subpopulation [87]. Conversely, a significant accumulation of both CD27+ memory and (to a lesser extent) CD27- naïve B cells have been described in the SS salivary glands [73, 87, 166], possibly as a result of increased migration/retention in the inflamed tissue, particularly in the context of ectopic lymphoid structures which develop in ~30% of SS salivary glands [141, 150, 181].

However, despite the evidence of profound peripheral and lesional B cell disturbances and humoral autoimmunity in SS, the mechanisms underlying the development of breach of self-tolerance and the onset of B cell autoreactivity in SS patients are still unclear.

In physiological conditions, self-reactive (and polyreactive) B cells, which are normally generated in the bone marrow as a consequence of random V(D)J recombination process, are silenced before entering the mature peripheral B cell compartments at two major tolerance checkpoints. The first occurs in the bone marrow between the early immature and immature B cell stage, while the second checkpoint between the transitional and the mature naïve B cell stage allowing the reduction of autoreactive/polyreactive B cells from the peripheral, circulating naïve pool [33, 182, 183]. Conversely, during autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), perturbation of these early B cell tolerance checkpoints have been described, as demonstrated by the increased frequency of polyreactive and self-reactive B cells in the naïve peripheral B cell compartment [42, 182].

However, whether similar defects in B cell differentiation tolerance checkpoint are present in SS patients is currently unknown. In this part of my PhD project I characterised the frequency of self- and poly-reactive circulating naïve B cells from patients with SS using a recently pioneered approach [33, 34] which allows the analysis of reactivity of the B cell receptor (BCR) of single B cells through the cloning and *in vitro* expression of complete (i.e., IgH+IgL chains) recombinant monoclonal antibodies which bear identical specificity to the original B cells (for more details see Chapter 2).

4.2 SS patients.

Out of 96 single CD3-CD19+CD27-IgD+ naïve B cell sorted from each of the 4 out of 12 SS patients (SS3, SS5, SS12, and SS13), I obtained successful sequences from a total of 119 different VH and JH regions (SS3=19, SS5=53, SS12=17, SS13=30), 73 Vκ and Jκ regions (SS3=17, SS5=31, SS12=2, SS13=23), and 36 Vλ and Jλ regions (SS3=13, SS5=10, SS12=10, SS13=3). Similarly, we obtained successful sequences from a total of 78 different VH and JH regions from 2 healthy donors (JH and HD2). Clones with matching and productive VH and VL products were used for downstream cloning and recombinant antibody expression. All productive VH and VL products displayed unmutated germ line sequences.

4.3 Peripheral B cell subpopulation disturbances in SS patients.

Previous studies analyzing B cell subpopulations in SS patients have shown a significantly reduction of circulating CD27+ memory B cells with accumulation of CD27- naïve B cells [82]. Thus, I first used 4-color flow cytometry to analyze the frequency of naïve (CD3-CD19+CD27-IgD+), class-switched memory B cells (CD3-CD19+CD27+IgD-) and unswitched memory B cells (CD3-CD19+CD27+IgD+) in 12 patients with SS (**Figure 4.1A**). This analysis confirmed that in our cohort of SS patients the frequency of circulating CD27- naïve B cells was increased compared to HD (mean±SD 68.5±16.0% versus 45.8±6.7%, respectively), whereas the frequency of CD27+ switched (6.0±5.2% versus 17.5±8.1%) and unswitched (4.9±6.6% versus 20.6±2.4%) memory B cells was significantly reduced in peripheral blood of the SS patients analyzed compared to controls (**Figure 4.1B**, including *p* values).

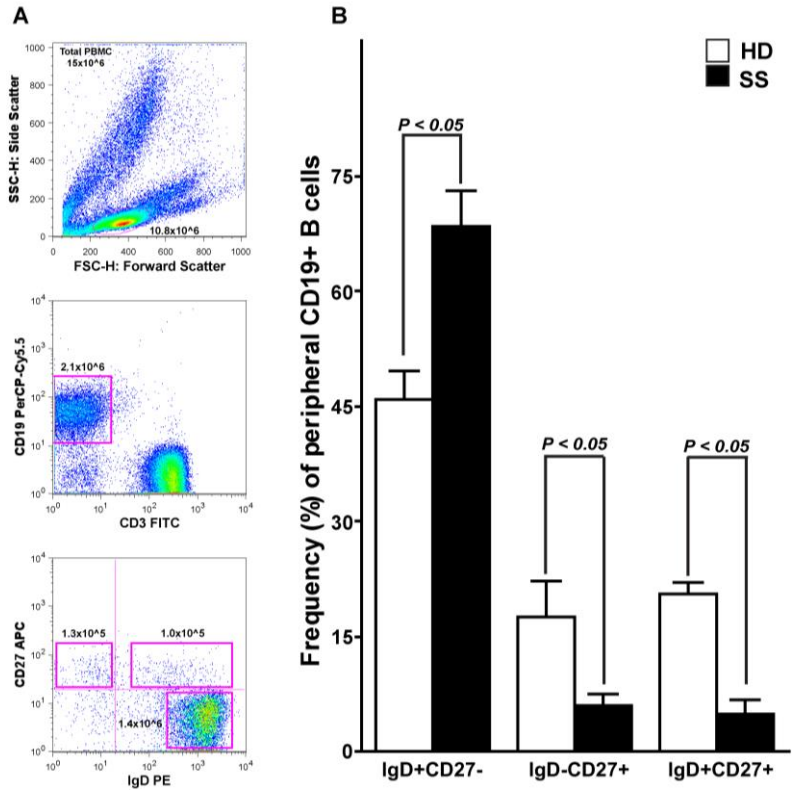

Figure 4.1 Isolation strategy of single naïve B cells and comparison of the frequencies of naïve, memory switched and unswitched B cells in SS patients and HD.

(A) PBMCs from SS patients were surface labeled with fluorochrome-coupled anti-CD19, anti-CD3, anti-IgD and anti-CD27. CD19+CD3- cells were gated and analyzed for IgD and CD27 expression. The sorting gate strategy for single CD19+CD3-CD27-IgD+ naïve, CD19+CD3-CD27+IgD+ unswitched memory, and CD19+CD3-CD27+IgD- class-switched memory B cells is shown. **(B)** The frequencies of peripheral naïve (CD3-CD19+CD27-IgD+), memory switched (CD3-CD19+CD27+IgD-) and unswitched (CD3-CD19+CD27+IgD+) B cells among all CD19+ B cells are shown. Differences between patients (*n=12*) and HD (*n=3*) were found to be statistically significant using the nonparametric Mann-Whitney U test (*p* value is reported over each graph). Error bars indicate standard error of the mean (SEM) for individual patient or control.

4.4 Immunoglobulin (Ig) gene repertoire characterization of naïve B cell antibodies cloned from SS peripheral blood B cells.

I studied four patients with a diagnosis of primary SS (SS3, SS5, SS12, and SS13) and two HD as control (JH [177] and HD2). All 4 SS patients displayed anti-Ro positivity, whereas 2 out of 4 reacted against La (Table 2.1). To characterise the Ig genes expressed by naïve B cells in these SS patients, I sorted CD3-CD19+CD27-IgD+ naïve B cells as single cells by flow cytometry from peripheral blood. The analysis of 119 V(D)J gene segments demonstrated a similar variable VH, D and JH gene repertoire in control and SS naïve B cells (**Figure 4.2A**). VH3 was expressed more frequently, as expected since it is the largest variable region family, followed by VH4 and VH1 both in SS patients and HD. I found a frequent usage of JH4 followed by JH6, JH5 and JH3 while JH1 and JH2 were expressed less frequently both in SS patients and controls, as previously shown [173]. Similarly, no significant differences in V and J gene usage for kappa (κ, 73 sequences) and lambda (λ, 36 sequences) chains were observed between SS patients and HD (**Figures 4.3A and 4.3B**). SS naïve Ig gene transcripts displayed a significantly higher frequency of positively charged IgH complementary determining regions 3 (CDR3s), a feature commonly associated with autoreactive antibodies [33] (**Figure 4.2B, bottom panel**). However, the frequency of long CDR3s, another feature frequently displayed by polyreactive antibodies [33], was similar between HD and SS patients (**Figure 4.2B, top panel**). Thus, I concluded that overall there are no major abnormalities in the Ig gene repertoire of circulating naïve B cells in SS patients as previously reported [82].

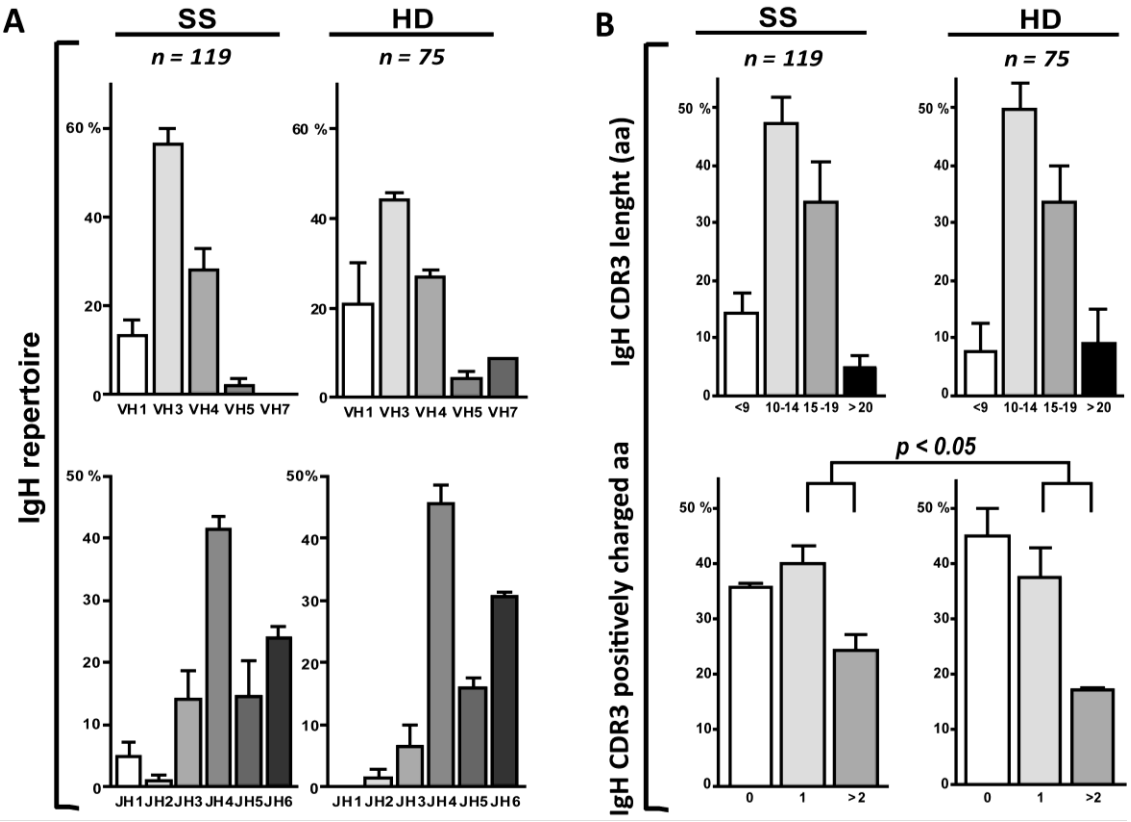

Figure 4.2 IgH gene analysis of naïve B cells from SS patients and HD.

Single naïve B cell antibodies from four SS patients and two HD [177] were analyzed for **(A)** IGH V family and J gene usage, and **(B)** IgH CDR3 aa length and positive charges. The absolute number of sequences analyzed is reported over each graph. Error bars in bar graphs indicate standard error of mean (SEM) for individual patient or control.

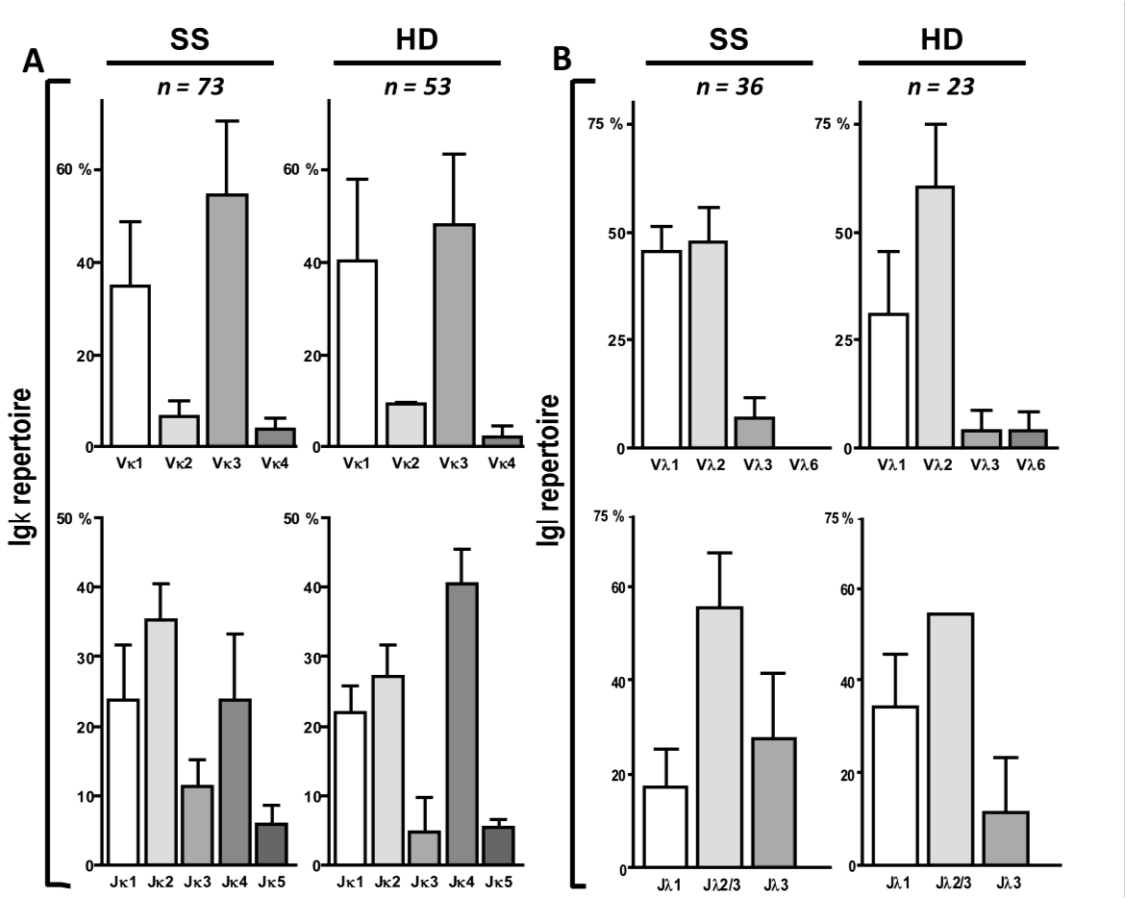

Figure 4.3 IgL gene analysis of naïve B cells from SS patients and HD.

Single naïve B cell antibodies from four SS patients and two HD were analyzed for IGK **(A)** and IGL **(B)** V family and J gene usage. The absolute number of sequences analyzed is reported over each graph. Error bars in bar graphs indicate standard error of mean (SEM) for individual patient or control.

Table 4.1 IgH and L gene repertoire analysis and reactivity of IgD+ single B cells from 4 SS patients. Clones highlighted in grey are those expressed as recombinant antibodies. (-) and (+), negative and positive charges within the heavy chain CDR3; length, number of amino acids within the CDR3; +, clones reactive in ELISA/IFA; -, clones non-reactive in ELISA/IFA; ND, not determined; C, cytoplasmic pattern; N, nuclear pattern.

Patient 3	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
1n.3	3-74	2-2	1P	1	VLQQPSPLYFDY	0	12	λ	2-14	1	0	SSYTSSSTLV	0	10	-	-	+	-	-	-	--	-	-
2n.3	3-30	6-6	6	1	DLYSSSPPGY	0	10	λ	2-11	1	0	CSYAGSYTYV	0	10	nd	nd	nd	nd	nd	nd	--	nd	nd
3n.3	3-66	5-12	3	2	PYSGYDPRGAFDI	1	13	λ	3-9	2/3	1	QVWDSSTVV	0	9	-	-	-	-	-	-	--	-	-
4n.3	4-61	3-9	4	2	GARTYYDILTGYSTFDY	1	17	κ	1D-39	1	0	QQSYSTPRT	1	9	-	-	+	-	+	+	C	+	-
5n.3								λ	1-51	1	1	GTWDSSLSAYV	0	11									
10n.3								λ	1-40	1	1	QSYDSSLSGSSYV	0	13									
11n.3	1-18	1-26	4	1	DLVYSGSYGY	0	10	κ	3-11	2	0	QQRSNWPPYT	1	10	-	-	-	-	nd	nd	--	-	-
16n.3	3-11	5-5	3	1	VGGGYQGAFDI	0	11	κ	3-15	1	0	QQYNNWPPWT	0	10	-	-	-	-	-	+	C	-	-
17n.3								κ	1-9	2	0	QQLNSYPYT	0	9									
19n.3	4-39	3-9	4	1	LRGGSPFLFDY	1	11	κ	3-15	1	0	QQYNNWPKT	1	9	-	-	-	-	-	-	--	-	-
20n.3	3-13	3-10	4	2	GNFDSYYYGSGSPFDY	0	16	κ	1-5	1	0	QQYNSYSTWT	0	10	-	-	-	-	-	-	--	-	-
22n.3								κ	1-5	5	0	QQYNSYSPIT	0	10									
24n.3	1-3	3-22	6	2	DDSSGYYY	0	8	κ	1D-33	2	1	QQYDNLPT	0	8	-	-	-	-	-	-	--	-	-
27n.3								λ	2-14	3	0	SSYTSSSTWV	0	10									
28n.3	3-64	3-22	3	1	SVVTMVVGGRSAFDI	1	16	κ	3-11	2	0	QQRSNWPPYT	1	10	+	-	-	-	+	-	--	+	+
31n.3								κ	2D-29	1	0	MQSIQLPPT	0	9									
32n.3	3-7	3-10	6	2	LPRDYYGSGSYLYYFDY	1	17	κ	3-15	4	0	QQYNNWPPLT	0	10	-	-	-	-	-	-	--	-	-
35n.3	3-7	3-9	4	2	DILTGYSVTFDY	0	12	κ	3-20	2	0	QQYGSSPMYT	0	10	-	-	-	-	-	-	--	-	-
36n.3	4-61	3-10	4	3	TEGDYYGSGSQRGFDY	1	16	κ	1-5	1	0	QQYNSYPWT	0	9	-	-	-	-	+	-	--	-	-
37n.3	4-61	4-17	6	3	DHGDRRYYYYYMDV	3	14	λ	2-14	2/3	0	SSYTSSSTLVV	0	11									
42n.3								κ	3-15	3	0	QQYNNWPFT	0	9									
43n.3								κ	1D-39	5	0	QQSYSTSIT	0	9									
44n.3								κ	1-17	2	0	LQHNSYPYT	1	9									
47n.3	3-30	3-9	4	3	EDYNSFDY	0	8	κ	1-27	3	0	QKYNSAPFT	1	9	-	-	-	-	-	+	C	-	-
50n.3								λ	2-14	3	0	SSYTSSWV	0	8									
54n.3	4-39	6-25	3	3	HDPGDLRGAFDI	2	12	λ	2-23	2/3	0	CSYAGSSTLV	0	10	-	-	-	-	-	+	C	-	-
68n.3	3-23	3-9	4	4	DRMGYFDYGDYFDY	1	14	λ	1-47	3	2	AAWDDSLSGWV	0	11	-	-	-	-	-	-	--	-	-
78n.3	3-13	6-19	3	3	RRGQWLGADDAFDI	2	14	λ	1-44	2/3	2	AAWDDSLNGVV	0	11	-	-	-	-	-	-	--	-	-
79n.3	3-7	2-2	4	1	TRSSTFD	1	7	λ	2-11	2/3	0	CSYAGSYTWV	0	10	-	-	-	-	-	-	--	-	-
91n.3	3-7	6-13	1	1	EGYSSSWHSRTGYSSTG	2	17	λ	1-51	1	1	GTWDSSLSAYV	0	11									

Patient 5	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
2n.5	4-59	4-23	4	1	GRTTVVRGNYFDY	2	13	λ	2-14	1	0	SSYTSSSTYV	0	10	-	-	-	-	nd	nd	--	nd	nd
3n.5	4-39	6-19	5	2	QKQWLVEYWFDP	1	12																
4n.5	3-7	5-5	4	0	VVYSYGYNY	0	9	κ	3-15	1	1	QQYNNWPPET	0	10	-	-	-	-	-	+	C+N	-	-
5n.5	4-61	3-22	3	3	DSWYYYDRGGAFDI	1	14	κ	3-20	3	0	QQYGSSPFT	0	9									
6n.5	3-21	5-5	5	2	NRGYSYGTDWFDP	1	13	λ	1-51	2/3	1	GTWDSSLSAVV	0	11									
8n.5	3-11	3-22	5	3	VDQSYYDSSGPHWWFDP	1	17	λ	1-51	2/3	1	GTWDSSLSAVV	0	11									
11n.5	1-18	2-8	6	2	VGSTGGDYYYYGMDV	0	15	κ	2-30	1	0	MQGTLART	1	8	-	-	-	+	-	-	--	-	-
12n.5	4-4	3-22	4	1	GISSGWAGKFDY	1	12	λ	2-14	2/3	0	SSYTSSSTRRVV	2	12	+	+	+	+	+	+	C+N	+	+
14n.5	3-30	6-13	6	2	SRERAAAVPGVVMSYGMDV	2	19	κ	1-9	3	0	QQLNSYFT	0	8	+	+	+	+	-	-	--	-	-
17n.5	3-30	5-5	4	4	DEGDTAMGPFDY	0	12	κ	1-5	1	0	QQYNSYWT	0	8	-	-	-	-	-	+	C	-	-
18n.5	3-30	6-13	4	1	DSYSSSPRGY	1	10																
19n.5	4-34	6-6	4	1	ESSSHLLGY	1	9	κ	2-28	2	0	MQALQTPRT	1	9									
20n.5	3-48	7-27	6	2	DIGINWGFYYYYYGMDV	0	17	κ	1-9	4	0	QQLNSYPPVT	0	10	+	-	-	-	+	+	C+N	-	+
22n.5	4-4	3-3	5	2	ETIFGVVIISNWFDP	0	15	κ	3-11	4	1	QQRSNWPPELT	1	11	-	-	-	-	-	-	--	-	-
23n.5	3-23	2-2	4	1	GPAIVVVPAAMPSWFDY	0	17																
24n.5	4-59	4-23	4	1	AVVLDY	0	6	κ	3-20	2	0	QQYGSSPQT	0	9	-	-	-	-	-	+	C	-	-
29n.5	1-24	4-23	2	1	ASRWYFDL	1	8	κ	3-11	3	0	QQRSNWPFT	1	9									
30n.5	3-30-3	3-3	5	1	VRFLEWLFT	1	9																
31n.5	3-30-3	3-22	4	3	DRRYYYDSSGYLDY	2	14																
32n.5	3-23	5-5	6	1	GTAMYYYYYGMDV	0	13																
33n.5	3-43	2-15	4	1	GGSCSGGSCYVGPDY	0	15	κ	1-39	3	0	QQSYSTPFT	0	9									
36n.5	4-34	5-5	4	1	ARYTAMAKCFDY	2	12																
37n.5	4-61	5-12	6	1	TATYYYYGMDV	0	11																
38n.5	3-30-3	2-15	3	2	EVDCSGGSCYSLAS	0	14	κ	2-28	4	0	MQALQTPVT	0	9									
40n.5	1-46	1-26	4	1	GKARSYFDY	2	9	κ	3-20	1	0	QQYGSSPTT	0	9									
41n.5	3-49	2-15	4	1	VLYCSGGSCHGAFDY	1	15	κ	1-33	2	1	QQYDNLPYT	0	9									
44n.5	3-48	3-22	4	3	VHLYYYDSSGYYYDDY	1	16	κ	3-11	4	0	QQRSNWPLLT	1	10	-	-	-	-	nd	nd	--	-	-
45n.5	4-b	3-16	5	2	VERIAGGALARIDP	2	14																
50n.5								κ	3-20	1	1	QQYGSSPET	0	9									
54n.5	3-30-3	5-5	6	2	DRGLSGYSYGHWAYYYGMDV	2	20	λ	1-51	2/3	2	GTWDSSLSAEVV	0	12	-	+	-	-	-	-	--	-	-
56n.5	4-34	6-19	5	2	RGKARYSSGWYKDNWFDP	4	18	λ	2-23	2/3	0	CSYAGSPV	0	8	-	-	-	-	+	-	--	+	+
59n.5	3-30	3-9	5	3	DPQNENWSPPYNWFDP	0	16	λ	1-44	1	2	AAWDDSLNGRV	1	11	-	-	-	-	-	-	--	-	-
60n.5	3-7	6-13	3	2	AKRQLDAFDI	2	10	κ	3-15	3	0	QQYNNWPSFT	0	10	-	-	-	-	-	-	--	-	-
61n.5	3-21	3-16	4	3	IDGLRLGELSLVRSPDC	2	17	κ	3-11	5	0	QQRSNWPPVT	1	10	-	-	-	-	+	-	--	-	-
62n.5	3-23	3-22	6	2	EGLIVVIIGGMDV	0	13	λ	1-44	2/3	2	AAWDDSLNGVV	0	11									
63n.5	3-30	6-6	6	2	DIAARSYYYYGMDV	1	14	κ	3-15	1	0	QQYNNWPRT	1	9									
64n.5	1-24	1-26	5	1	LPWDSGSHGGLV	1	12																
66n.5	4-39	4-17	4	4	DHPLYDYGDLGPRVLFDY	2	18	λ	1-44	2/3	2	AAWDDSLNGVV	0	11									
67n.5	4-61	1-7	5	1	APRVRVGLDS	2	10	κ	3-20	2	0	QQYGSSPLT	0	9	-	+	-	-	-	+	C	-	-
68n.5	4-59	5-24	4	1	AGLGYRRYYFDY	2	12	κ	2-29	2	0	MQSIQPYT	0	8									
69n.5	1-2	5-18	6	2	TYGDGMDV	0	8	κ	3-20	1	0	QQYGSSLWT	0	9	-	-	-	-	-	+	C	-	-
72n.5	4-59	6-6	5	1	VGVAARPGGWFDP	1	13	κ	4-1	2	0	QQYYSTPYT	0	9	nd	nd	nd	nd	nd	nd	--	nd	nd
73n.5	5-51	4-17	4	3	RDGDYPFDY	1	9	κ	4-1	4	0	QQYYSTPRLT	1	10	-	-	-	-	-	-	--	-	-
79n.5	3-30-3	4-17	3	3	GDYGDSPDI	0	9	κ	1-5	1	0	QQYNSYSRT	1	9									
80n.5	3-7	2-21	4	0	GLWWCTY	0	7	κ	2-28	2	0	MQALQTPYT	0	9									
83n.5	4-b	4-4	2	1	PGKVTTHYWYFDL	2	13	λ	2-14	2/3	0	SSYTSSSTLV	0	10	+	-	-	-	-	-	--	-	-
84n.5	4-4	5-24	5	1	GRDGYNYLG	1	9																
85n.5	3-30	6-19	5	2	DIAVAGKGGWFDP	1	13	κ	3-11	1	0	QQRSNWPWT	1	9									
86n.5	4-61	2-8	4	1	TSTNPFLFDY	0	10	κ	1-5	4	0	QQYNSFPLT	0	9									
89n.5	3-11	2-2	6	2	DRNIVPYYYYGMDV	1	14	κ	3-20	2	0	QQYGSSPYT	0	9									
90n.5	4-30-2	3-16	5	3	ERGDRGVITRHGWFDP	4	16																
91n.5	4-b	3-3	6	3	LPPVLRFLEWFTPGDYYYGMDV	1	22	κ	1-39	4	0	QQSYSTPLT	0	9									
93n.5	4-b	3-22	4	2	PTYYYDSSGYWAYYFDY	0	17	κ	3-11	4	0	QQRSNWPLT	1	9									
95n.5	4-b	5-5	6	1	QGQLWLPPTSHGMDV	1	15																

Patient 12	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
3n.12	3-33	4-17	4	1	VGGTTTVTSTTGYYFDY	0	17	λ	3-21	3	2	QVWDSSSDHLWV	1	12	-	-	-	-	-	-	--	-	-
25n.12	3-21	5-5	4	1	SGPNARGYSYGFDY	1	14																
26n.12	3-15	6-19	4	2	EGGQQWLVRGYYFDY	1	15	λ	1-51	2/3	1	GTWDSSLSVL	0	10	-	-	-	-	-	+	C	-	-
37n.12	3-23	3-9	4	2	GRSGRRGYFDWLGIDY	3	16	λ	2-14	2/3	0	SSYTSSSTLGVV	0	12	-	-	-	-	-	-	--	-	-
38n.12	4-34	3-3	6	2	YDFWSGYYPTAYYYYGMDV	0	19																
50n.12	4-59	3-10	4	1	ATPSPGVTMVRGVIRGGYFDY	2	21																
55n.12	3-33	2-15	6	2	DVAVVVVNYYYYGMDV	0	16	λ	1-51	2/3	1	GTWDSSLSAV	0	10	+	-	-	-	nd	nd	--	-	-
56n.12	3-21	4-17	1	0	HSTTVTTLRYFQH	3	13	κ	3-11	4	0	QQRSNWPLT	1	9	-	-	+	-	+	+	C	-	-
60n.12	1-69	2-15	5	3	DGRSGGGDWFDP	1	12	κ	3-20	2	0	QQYGSSPRT	1	9									
61n.12	3-15	2-8	4	1	DGVVMVYAIGY	0	11	λ	1-47	2/3	2	AAWDDSLSVVV	0	11	+	-	-	-	-	+	C	-	-
72n.12	4-59	6-25	4	1	GSAPPGGYFDY	0	11	λ	2-11	1	0	CSYAGSYTYV	0	10									
82n.12	3-21	6-13	4	4	DAPGDRSGESIAAAPDY	1	17	λ	1-47	2/3	2	AAWDDSLSGVVF	0	12	-	-	+	-	-	+	C	-	-
84n.12								λ	3-25	2/3	1	QSADSSGTYRV	1	11									
85n.12								λ	2-11	2/3	0	CSYAGFVV	0	8									
92n.12	1-2	6-6	5	1	GGYSSSRFDP	1	10	λ	1-44	3	2	AAWDDSLNGPV	0	11									
95n.12	1-69	4-23	3	2	DPATSFDI	0	8																
96n.12	1-69	4-23	6	3	GGRGLPWEPDYYYYGMDV	1	18																

Patient 13	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
19n.13	4-34	4-23	1	1	GAFGGNSVDFQH	1	12	κ	1-39	1	0	QQSYSTPRT	1	9	+	-	-	-	-	+	C	-	-
30n.13	1-69	1-14	3	2	HDNRGPFDI	2	9																
31n.13	3-66	3-22	3	1	VGRITIQSRAFDI	2	13	κ	1-13	4	0	QQFNSYPHRLT	2	11									
43n.13	4-39	3-10	5	3	HSMVRGVDVDNWFDP	2	15																
56n.13	3-53	3-22	4	3	LRYYDSKGDFDY	2	12																
66n.13	3-23	3-22	4	2	EHRITMIVVLIYYFDY	2	16	κ	1-39	1	0	QQSHSTPKT	2	9	-	-	-	-	-	+	C	-	-
67n.13	3-30	3-3	4	2	DRAGEWLLFST	1	11	κ	1-39	3	0	QQSYSTPLT	0	9									
68n.13	5-a	6-6	3	3	PEYSSSSIPILDAFDI	0	16						0										
72n.13	3-43	3-10	4	1	ARHYYGSGSYLDY	2	13	κ	3-15	1	0	QQYNNWPPRT	1	10	-	-	-	-	-	+	C	-	-
78n.13								λ	1-36	3		-											
79n.13	4-61	1-7	4	1	GSWNYADY	0	8	κ	3-15	1	0	QQYNNWPGWT	0	10	-	-	-	-	-	+	C	-	-
83n.13	3-53	5-12	5	1	VQWGGYGLVSNWFDP	0	15	κ	1-39	4	0	QQSYSTPALT	0	10									
88n.13								λ	1-44	3	2	AAWDDSLNGPV	0	11									
89n.13								λ	2-14	3	0	SSYTTINTL	0	9									
5n.13	4-34	2-2	6	1	YCSSTSCPRPYYYYGMDV	1	18	κ	1-39	5	0	QQSYSTLPIT	0	10	-	-	-	-	+	+	C+N	-	+
24n.13	1-46	2-21	5	0	CTGHRLWWCPPRW	3	13	κ	1-5	2	0	QQYNSSPYT	0	9	-	-	+	-	+	+	C	-	-
30n.13	3-23	2-15	6	2	GGYCSGGSCYRDYYYYGMDV	1	20	κ	1-5	1	0	QQYNSYPTWT	0	10	-	-	-	-	-	-	--	-	-
32n.13	1-8	2-15	5	1	TRSGGGSAGTFDP	1	13	κ	1-5	1	0	QQYNSYWT	0	8	-	-	-	-	-	-	--	-	-
36n.13	4-59	3-3	6	3	DSRFLEWPYGMDV	1	13	κ	3-11	2	0	QQRSNWPPYT	1	10	-	-	-	-	-	+	C	-	-
43n.13	3-43	6-19	4	2	GESSGWYYFDY	0	11	λ	2-14	2/3	0	SSYTSSSTLV	0	10	-	-	-	-	-	-	--	-	-
48n.13	4-34	6-13	6	1	GKGSWPYYGMDV	1	12	κ	1-17	3	1	LQDYNYPPFT	0	10	nd	nd	nd	nd	nd	nd	--	nd	nd
56n.13	4-59	4-23	4	4	EMDYGGNPREFDY	1	13	κ	3-11	5	0	QQRSNWPPIT	1	10	-	-	-	-	-	-	--	-	-
59n.13	3-11	1-26	4	1	IPGNMGATGLDY	0	12	κ	2-30	2	0	MQGTHWPYS	1	9	-	-	-	-	-	+	C	-	-
69n.13	4-59	3-22	4	1	GITQYYFDY	0	9	κ	3-15	2	0	QQYNNWPPYT	0	10	nd	nd	nd	nd	nd	nd	--	nd	nd
70n.13	3-23	3-3	6	5	DHFFGDFWSGYYDLDYYYGMDV	1	22				0												
71n.13	5-51	6-25	5	1	SSGGTWFDP	0	9	κ	4-1	3	0	QQYYSTPFT	0	9	-	-	+	-	-	-	--	-	-
79bn.13	3-21	3-22	5	1	AAHYYDSSGPR	2	11	κ	3-15	2	1	QQYNNWPPDS	0	10	-	-	-	-	+	+	C+N	-	-
81n.13	3-9	6-19	4	1	GSGWSYFDY	0	9	κ	1-5	1	0	QQYNSYSWT	0	9	nd	nd	nd	nd	nd	nd	--	nd	nd
82n.13	3-11	1-26	6	2	DKVPVGALGGMDV	1	13	κ	1-33	2	1	QQYDNLYS	0	8	-	-	-	-	-	-	--	-	-
90n.13	3-30	2-2	6	2	DLNSYCSSTSCYPSYYYYGMDV	0	22																
91n.13	3-21	3-16	3	2	GPLSLGEPGAFDI	0	13	κ	1-5	2	0	QQYNSAPVYT	0	10	nd	nd	nd	nd	nd	nd	--	nd	nd
94n.13	3-30	6-19	4	3	DSDFQIAMGSGWYLDY	0	16	κ	1-8	4	0	QQYYSYPLT	0	9									
95n.13	3-7	6-25	3	2	DRSGRAFDI	2	9	κ	4-1	3	0	QQYYSTPL	0	8	-	-	-	-	-	+	N	-	-

Table 4.2 IgH and L gene repertoire analysis and reactivity of IgD+ single B cells from 2 healthy donors. Clones highlighted in grey are those expressed as recombinant antibodies. (-) and (+), negative and positive charges within the heavy chain CDR3; length, number of amino acids within the CDR3; +, clones reactive in ELISA/IFA; -, clones non-reactive in ELISA/IFA; ND, not determined; C, cytoplasmic pattern; N, nuclear pattern.

HD2	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
6n.HD2	3-23	2-2	6	2	DGGLHCSSTSCPSLFYYGMDV	1	21	κ	2-40	1	1	MQRIEFPWT	1	9	-	-	-	-	-	-	--	-	-
7n.HD2	1-69	2-15	4	3	DDCSGGSCYFDY	0	12																
10n.HD2	5-51	6-13	4	2	PRGYRSSSYEAFYFDY	2	16	κ	3-11	4	0	QQRGNWPRLT	2	10	-	-	-	-	-	-	--	-	-
16n.HD2	3-23	3-22	3	3	HLSRDSSVLDAFDI	2	14	κ	1-5	2	0	QQYNSYWGA	0	9									
20n.HD2								κ	1-33	4	1	QQYDNLALT	0	9									
22n.HD2	4-34	2-8	3	1	AKVYASAFDI	1	10	κ	3-20	1	0	QQYGSSPWT	0	9	-	-	-	-	-	-	--	-	-
23n.HD2	3-7	3-16	3	3	EDWGAFDI	0	8	κ	1-5	1	0	QQYNTYSPWT	0	10	-	nd	nd	nd	nd	nd	--	-	-
24n.HD2	4-39	2-21	4	2	LGVGKMGAYCGGDCYSLFDY	1	20	κ	1-27	3	0	QKYNSAPH	2	8									
29n.HD2	3-64	6-13	6	2	FGIAAAGRDYYYGMDV	1	16	κ	3-15	1	0	QQYNNWPPWT	0	10	nd	nd	nd	nd	nd	nd	--	-	-
30n.HD2	4-34	2-2	6	1	ARCSSTSCYLRNYYYYYGMDV	2	21	κ	1-39	5	0	QQSYSTPPT	0	9									
31n.HD2	3-21	3-10	4	1	SFYGSGTDY	0	9	λ	1-51	2/3	1	GTWDSSLSAGV	0	11	-	-	-	-	-	+	C	-	+
32n.HD2	3-73	3-10	5	1	TTGFGELF	0	8	κ	1-39	2	0	QQSYSTPLT	0	9									
33n.HD2	1-69	3-10	6	2	LPYGSDYYYGMDV	0	13	λ	2-11	1	0	CSYAGSYTVYV	0	11	-	+	-	-	nd	nd	--	nd	nd
35n.HD2	3-74	6-19	3	3	DSSGWNDAFDI	0	11	κ	1-39	4	0	QQSYSTPPT	0	9	-	-	-	-	-	-	--	-	-
36n.HD2								κ	3-20	4	0	QQYGSSPLT	0	9									
41n.HD2	3-23	3-16	4	2	YRAVGYDYVWGSYRTNYFDY	2	20	κ	1-27	4	0	QKYNSAPHT	2	9									
43n.HD2	4-39	3-3	5	1	QTRITIFGVVILGWFDP	1	17	λ	2-14	1	0	SSYTSTSTRV	1	10									
44n.HD2	1-46	6-19	4	1	ARGIAVAGTLDY	1	12	λ	2-14	1	0	SSYTSSSTFYV	0	11	-	-	-	-	-	-	--	-	-
45n.HD2	4-61	6-19	4	2	IAVPGNRRSEAFDF	2	14																
47n.HD2								κ	2-28	4	0	MQALQTPL	0	8									
48n.HD2	4-39	5-24	5	2	RDGYNFWFDP	1	10	λ	2-23	2/3	0	CSYAGSSTVV	0	10									
55n.HD2	1-69	3-22	4	3	DPNYKTYYYDSSGYYYVPVYFDY	1	23	λ	1-44	2/3	2	AAWDDSLNGVV	0	11	-	-	-	-	nd	-	--	-	-
56n.HD2	1-69	6-13	4	0	VGIAAAGSSY	0	10	κ	3-15	5	3	QQYNNWPVEA	3	11	-	-	-	-	-	-	--	-	-
58n.HD2	1-3	5-24	5	2	DLGTPGAPFDP	0	11	λ	1-44	2/3	3	AAWDDSLNGQDVV	0	13									
59n.HD2	4-59	6-13	6	1	YGAAGPTRFYYYGMDV	1	16	κ	1-17	1	1	LQDYNYPPT	0	9									
60n.HD2	1-69	3-9	6	2	GYYDILTGPIAYYYYGMDV	0	19	κ	1-39	2	0	QQSYSTPT	0	8									
65n.HD2	3-74	6-19	5	2	EVAVAGQGWFDP	0	12	κ	1-39	2	0	QQSYSTPPT	0	9									
67n.HD2	1-18	3-16	6	1	LGLMGPRNTPGGWVYGMDV	1	19																
68n.HD2	4-61	3-22	4	1	ISRSTFDY	1	8	λ	3-21	2/3	2	QVWDSSSDHPV	1	11									
69n.HD2	1-69	2-2	6	1	ACSSTSCYTNYYYGMDV	0	17	κ	1-5	1	0	QQYNSYST	0	8									
71n.HD2	3-23	1-7	6	1	TFRYNWNYFSGSRPGYYGMDV	2	21	κ	1-39	4	0	QQSYSTPLT	0	9									
72n.HD2	1-69	4-17	4	3	WGGDYQAYGDYAVHFFDY	1	18	κ	3-15	2	0	QQYNNWPYT	0	9									
78n.HD2	3-23	3-10	4	1	PRRGLLWFGELPHY	3	14	κ	1-9	4	0	QQLNSYPLT	0	9									
79n.HD2	3-21	6-13	4	1	GYSSSCFDY	0	9	κ	1-5	4	0	QQYNSYSP	0	8									
80n.HD2	3-20	3-22	4	2	NYYDSSGYYYFDY	0	13	κ	1-39	3	0	QQSYSTPIFT	0	10									
81n.HD2								κ	3-11	1	0	QQRSNWPPWT	1	10									
82n.HD2	1-3	6-19	4	1	VWYSSGWYDY	0	10	κ	3-11	2	0	QQRSNWPPYT	1	10									
84n.HD2	3-33	6-13	4	2	DQSGGAAAGFDY	0	12																
87n.HD2								κ	3-15	1	0	QQYNNWPSWT	0	10									
88n.HD2	4-59	3-9	6	1	GAGLFLNYYYYGMDV	0	15	λ	1-44	1	2	AAWDDSLNGSYV	0	12									
89n.HD2	4-4	3-10	5	1	GGSHYGSGSYYNWFDP	1	16	κ	1-39	3	0	QQSYSTPLG	0	9									
90n.HD2	3-21	2-2	6	1	SCSSTSCLYGMDV	0	13	λ	1-47	2/3	2	AAWDDSLSGWV	0	11									
91n.HD2	3-33	6-19	4	2	DPPYSSGWYFDY	0	12	λ	2-14	1	0	SSYTSSSTFYV	0	11									
93n.HD2	1-24	6-13	5	2	SGSSWDNWFDP	0	11	κ	3-11	4	0	QQRSNWPPT	1	9									
94n.HD2	3-11	3-9	6	2	MGYDILTAYYYYGMDV	0	16	κ	1-39	2	0	QQSYSTPYT	0	9									

JH(*)	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
JH.1 D4	7-81	3-10	4	2	GDITMVRVTLADY	1	13	κ	3-20	1	0	QQYGSSAWT	0	9									
JH.1 E3	4-34	1-26	6	1	GSYRPPLVYYYYGMDV	1	16	κ	3-20	4	0	QQYGSSPLT	0	9	-	-	-	-	nd	nd	--	nd	nd
JH.1 F5	3-30	4-17	4	2	DLFTVTHFDY	1	10	κ	3-15	4	0	QQYNNWPPLT	0	10	-	-	-	-	-	-	--	-	-
JH.1 F8	3-21	3-22	6	2	VGAVAGGVDYYYYYGMDV	0	18	κ	2D-28	4	0	MQALQTLALT	0	10	-	-	-	-	-	-	--	-	-
JH.1 G8	3-48	1-26	6	1	LSGSYSNYYYGMDV	0	14	κ	1D-12	4	0	QQANSFPLT	0	9	-	-	-	-	-	-	--	-	-
JH.1 G10	3-23	2-2	4	4	EGPFDIVVVPAALDFDY	0	17	κ	3-15	2	0	QQYNNWPSYT	0	10	-	-	-	-	-	+	C	-	-
JH.1 H5	4-59	/	4	2	LGKMDLSPFDY	1	11	κ	3-20	2	0	QQYGSSPRT	1	9	-	-	-	-	-	-	--	-	-
JH.1 H11	3-33	/	6	2	DHSVTPYLYYYYGMDV	1	16	κ	2D-28	1	0	MQALQTPRT	1	9	+	+	-	+	-	-	--	-	-
JH.2 A2	3-74	1-26	6	3	DPVWELFSAASGGAHYYYGMDV	1	22	κ	1D-39	2	0	QQSYSTPPT	0	9	-	-	-	-	-	-	--	-	-
JH.2 A8	3-7	6-19	1	1	DLSGQWLVRGRH	3	12	κ	1-8	4	0	QQYYSYPLT	0	9	-	-	-	-	-	-	--	-	-
JH.2 A9	4-31	/	5	3	DTAAETGNNWFDP	0	13	κ	3-11	5	0	QQRSNWPIT	1	9	-	-	-	-	-	+	C	-	-
JH.2 A11	4-61	/	4	2	DGKGLGYFDY	1	10	κ	4-1	1	0	QQYYSTPWT	0	9	-	-	-	-	-	-	--	-	-
JH.2 B5	7-81	3-22	4	4	VPPPNYYATVEEEYYFDY	0	18	κ	3-20	4	0	QQYGSSPRLT	1	10	-	+	-	-	nd	nd	--	nd	nd
JH.2 C3	3-23	3-10	5	1	GAAALLWFGEI	0	11	κ	3-20	2	0	QQYGSSPMYT	0	10	-	-	-	-	-	-	--	-	-
JH.2 C6	3-23	3-22	4	2	VRGLRPADYYFDY	2	13	κ	1-9	2	0	QQLNSYPYT	0	9	-	-	-	-	-	-	--	-	-
JH.2 C10	4-59	3-22	4	2	GPYYYDFDY	0	9	κ	3-15	1	0	QQYNNWPRT	1	9	-	-	-	-	-	+	C	-	-
JH.2 D7	3-48	/	6	2	EYRLARYYYYGMDV	2	14	κ	3-15	1	0	QQYNNWPYT	0	9	-	-	-	-	-	-	--	-	-
JH.2 E3	1-18	1-20	5	3	EPPGPNDVRNDP	1	12	κ	3-20	4	0	QQYGSSPFA	0	9	-	-	-	-	nd	nd	--	nd	nd
JH.2 E5	4-39	6-6	6	1	HPRIAAQVYGMDV	2	13	κ	1D-39	4	0	QQSYSTLPLT	0	10	-	-	-	-	-	+	C	-	-
JH.2 E8	3-9	6-19	5	1	GSHTSSSGWYDY	1	12	κ	3-15	2	0	QQYNNWPMYT	0	10	-	-	-	-	-	-	--	-	-
JH.2 F3	7-81	2-15	5	3	AEDIVVVVAAAGFDP	0	15	κ	3-20	4	0	QQYGSSPRT	1	9	-	-	-	-	nd	nd	--	nd	nd
JH.2 F9	5-51	6-13	3	1	QYSSRLRGGGAFDI	2	14	κ	3-20	4	0	QQYGSSLT	0	8	-	-	-	-	nd	nd	--	nd	nd
JH.1 B11	3-23	3-10	6	1	GATMVRGSLGMDV	1	13	λ	2-14	3	0	SSYTSSSTWV	0	10	-	-	-	-	-	+	C	-	-
JH.1 C12	4-59	2-2	6	1	ASPAAIGLPYYYYGMDV	0	17	λ	1-40	2/3	1	QSYDSSLSGFVV	0	12	-	-	-	-	-	-	--	-	-
JH.1 D2	1-18	4-17	4	3	ETGNSDYWAAPLDY	0	14	λ	2-8	1	0	SSYAGSNNPYV	0	11	-	-	-	-	-	-	--	-	-
JH.1 D10	4-59	6-13	4	1	HLPRIAAAAYDY	2	12	λ	2-14	1	0	SSYTSSSTYV	0	10	-	-	-	-	-	-	--	-	-
JH.1 F6	1-8	1-20	6	2	GMGLEPYSTIYGMDV	0	15	λ	2-14	2/3	1	SSYTSSSTLDVV	0	12	-	-	-	-	-	+	C	-	-
JH.1 H6	3-9	6-13	4	2	DMLRLSYSSSWYGAFDY	1	17	λ	2-11	3	0	CSYAGSYTLGV	0	11	-	-	-	-	nd	nd	--	nd	nd
JH.2 B12	3-23	6-19	4	2	DPLILATLGSSGFGPFFDY	0	19	λ	2-14	2/3	0	SSYTSSSTLYVV	0	12	-	-	-	-	nd	nd	--	nd	nd
JH.2 C12	4-4	/	6	1	GLTGSRQTPYYYYGMDV	1	17	λ	2-14	2/3	0	SSYTSSSTLYVV	0	12	-	-	-	-	-	+	C	-	-
JH.2 D8	3-21	3-9	4	3	DLFGGVDILTYFDY	0	14	λ	6-57	3	1	QSYDSSNHRV	2	10	-	-	-	-	-	-	--	-	-
JH.2 E6	3-33	2-2	4	1	AYCSSTSCYMRDY	1	13	λ	2-11	2/3	0	CSYAGSSVV	0	9	-	nd	nd	nd	nd	nd	--	nd	nd
JH.2 F6	1-2	6-19	4	1	DPPASYSSGWYGKLGY	1	16	λ	2-14	2/3	1	SSYTSSSTLDGV	0	12	-	-	-	-	-	-	--	-	-
JH.2 G12	5-51	3-22	5	2	GRSYYDSSGYYLQAGWFDP	1	19	λ	2-14	2/3	0	SSYTSSSTLYVV	0	12	-	-	-	-	nd	nd	--	nd	nd
JH.2 H4	4-59	3-22	2	4	LLTGADDSSGYYDWYFDL	0	18	λ	1-40	1	1	QSYDSSLSGRHV	2	12	-	nd	nd	nd	nd	nd	--	nd	nd

4.5 Accumulation of autoreactive naïve B cells in the peripheral blood of SS patients suggest impairment of early tolerance checkpoints in SS.

Out of all single naïve B cells isolated from the four SS patients, I managed to obtain matching IGH, IGK and IGL chains genes of 66 naïve B cells which were then cloned into specific expression vectors and used to generate monoclonal antibodies *in vitro* (SS3 = 19; SS5 = 20; SS12 = 7; SS13 = 20). As control, I expressed 45 monoclonal antibodies from the 2 HD (JH = 34 [177]; HD2 = 11) (for full details of the repertoire and reactivity of the naïve antibodies from SS patients and controls see Tables 4.1 and 4.2). As expected, all naïve B cells were characterized by no mutation in their variable Ig region showing germline Ig VH and VL genes.

I used these antibodies to determine the frequency of polyreactivity and self-reactivity in the naïve B cell compartment of SS patients and controls.

Antibody polyreactivity was tested using ELISAs against ssDNA, dsDNA, LPS and insulin at 1μg/ml. As shown in **Figure 4.4**, naïve B cell antibodies from SS patients showed very low levels of polyreactivity which was similar to HD (3.3% vs 2.4%), as previously reported [33]. Only one antibody (SS 12n5) was found to be highly polyreactive against all four antigens (**Figure 4.4**).

Conversely, the SS naïve antibodies were more frequently reactive towards dsDNA compared to controls (13.3% versus 2.3%, respectively, $p<0.05$, **Figure 4.4**), suggesting accumulation of autoreactive B cells. Autoreactive antibodies from SS naïve B cells generally displayed lower binding to dsDNA compared to highly polyreactive

antibodies from memory B cells of SLE patients such as JB40 and ED38 [34]. Thus, in order to confirm the true autoreactivity of these antibodies, I next investigated the frequency of self-reactive naïve B cells from SS patients using the human epithelial cell line Hep-2 by IFA, a commonly used test for ANA in clinical diagnostic, as previously reported [33, 34]. Positive results were further classified according to the Hep-2 staining pattern as anti-nuclear, anti-cytoplasmic and mixed anti-nuclear/anti-cytoplasmic antibodies. Overall, antibodies from SS patients displayed a significantly higher prevalence of immunoreactivity to Hep-2 compared to antibodies from HD (43.1% versus 25%, $p<0.05$, **Figure 4.5B**). The majority of antibodies from naïve B cells of SS patients displayed a cytoplasmic pattern while fewer antibodies showed antinuclear + cytoplasmic (8.6%) and antinuclear reactivity (1.7%) (**Figures 4.5A and 4.5B**). Conversely, among the 25% antibodies from HD showing positive ANA staining, none displayed a nuclear Hep-2 pattern.

Overall, these data demonstrated, both by ELISA and IFA, that autoreactive circulating naïve B cells accumulate in the peripheral blood of SS patients.

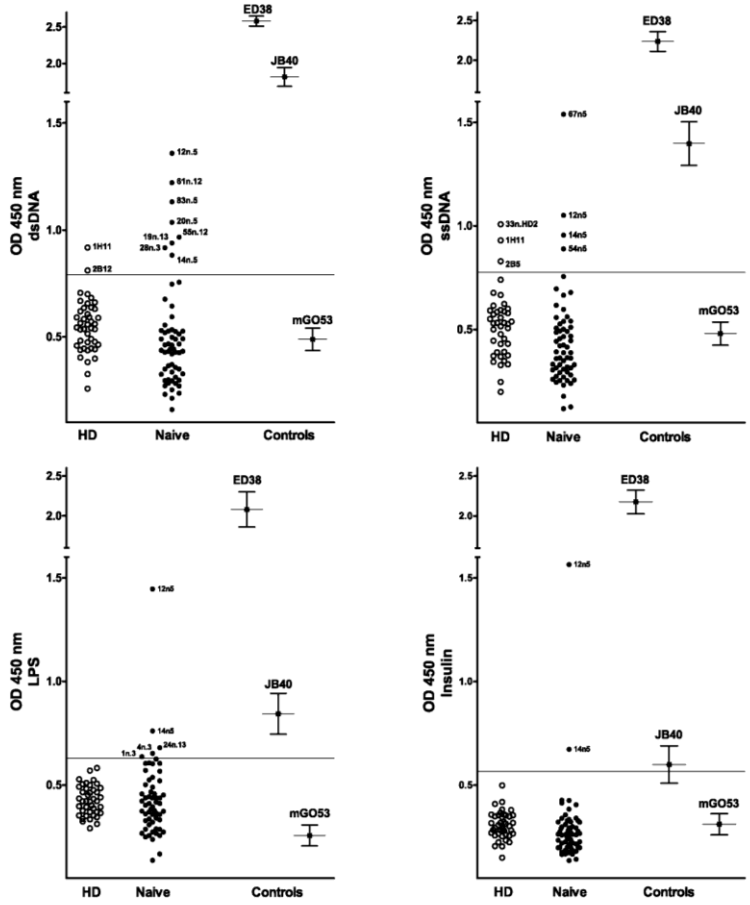

Figure 4.4 Polyreactivity of naïve B cell antibodies from SS patients and HD.

Naïve B cell antibodies from SS patients (*n=60*) and HD (*n=41*) were tested for reactivity with dsDNA (top left), ssDNA (top right), LPS (bottom left) and insulin (bottom right) by ELISA. Each graph shows the reactivity at a concentration of 1 μg/ml and it shows the result of two independent experiments. The cut-off OD (450 nm) at which antibodies were considered reactive is shown by the horizontal lines. Data points represent individual antibodies. Internal controls for polyreactivity (square dots) are shown in each graph and include mGO53 (negative;[34]), JB40 (low polyreactive;[34]), and ED38 (highly polyreactive;[34]). Error bars indicate standard error of mean (SEM) for the internal controls.

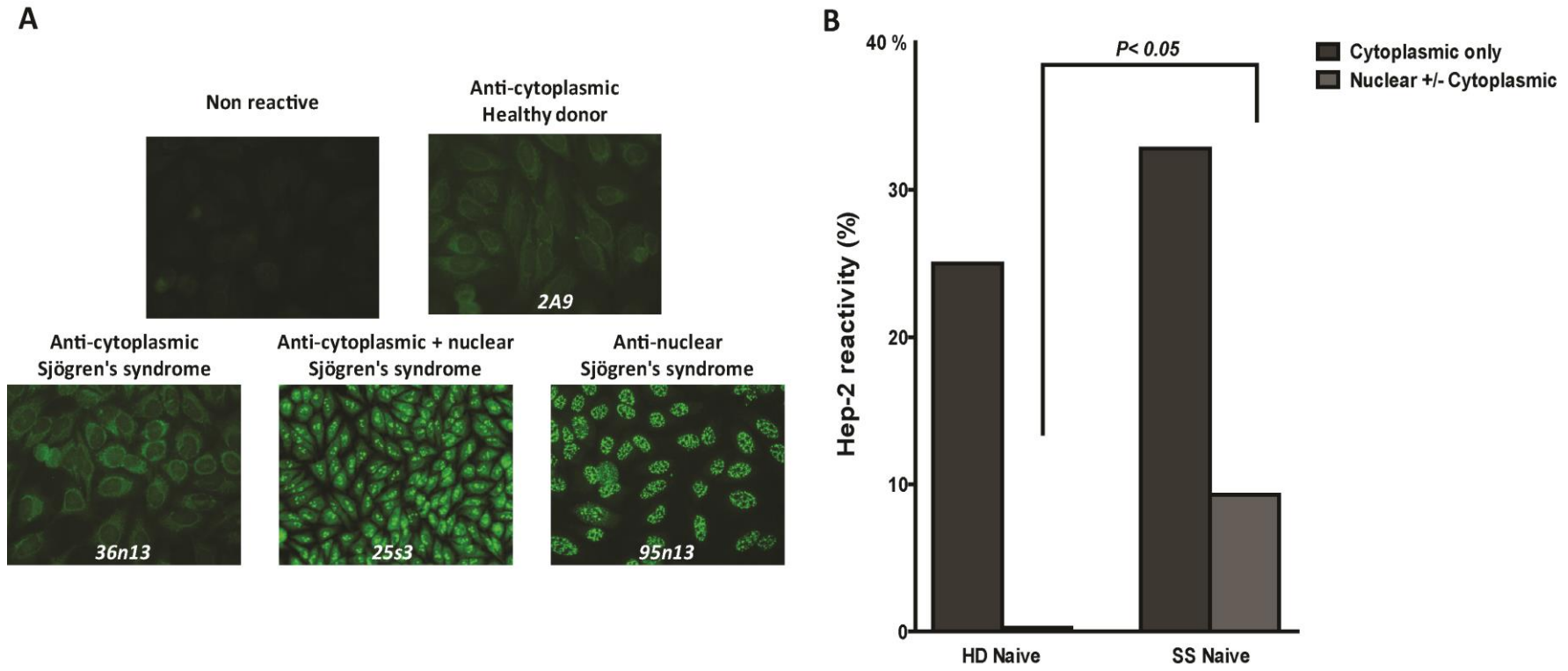

Figure 4.5 Hep-2 cell IFA self-reactivity of naïve B cell antibodies from SS patients and HD.

Naïve B cell antibodies from SS patients (n=58) and HD (n=32) were tested for self-reactivity by Hep-2 cell IFA assay. **(A)** Examples of cytoplasmic, cytoplasmic and nuclear, and nuclear Hep-2 cell staining pattern are shown. **(B)** Graph bars summarize the frequency of Hep-2 cell reactive antibodies with cytoplasmic, nuclear and/or cytoplasmic reactivity in SS patients and HD. *P* value compares ANA+ (nuclear ± cytoplasmic reactive) clones from SS patients and HD.

4.6 Presence of naïve B cells reactive against extractable nuclear antigens (ENA) in SS patients.

We then investigated the self-reactivity of the SS naïve B cell antibodies against ENA first using a general ENA screening ELISA, which includes the main antigens Sm, RNP, Ro/SSA, La/SSB, Scl-70 and Jo-1. Using this approach we observed that SS naïve B cell antibodies showed a significant increase in the frequency of ENA auto-reactivity compared to HD (19.6% vs 0%, $p < 0.01$, **Figure 4.6A**).

Next, we analysed whether some of these ENA-reacting SS antibodies were directed against Ro/SSA and/or La/SSB, which are part of the current classification criteria for pSS [48], using a specific diagnostic ELISA test. As shown in **Figure 4.6**, we observed reactivity to Ro/SSA (**Figure 4.6B**) and/or La/SSB (**Figure 4.6C**) in 6 out of 58 SS naïve B cell antibodies. Importantly, all the 6 clones positive for Ro/SSA and/or La/SSB gave matched positive results in the ELISA screening (**Figure 4.6**), confirming their reactivity. Interestingly, one of the Ro/SSA positive clone showed a purely cytoplasmic pattern at Hep-2 staining, confirming early evidence that anti-Ro antibodies do not necessarily display the classical speckled nuclear pattern but can react with a fibrous cytoplasmic network similar to cytokeratin [91].

Of relevance, one of the SS antibody that showed high levels of reactivity against Ro/SSA and La/SSB (SS 12n5), also recognized ssDNA, dsDNA, LPS and insulin and was therefore polyreactive.

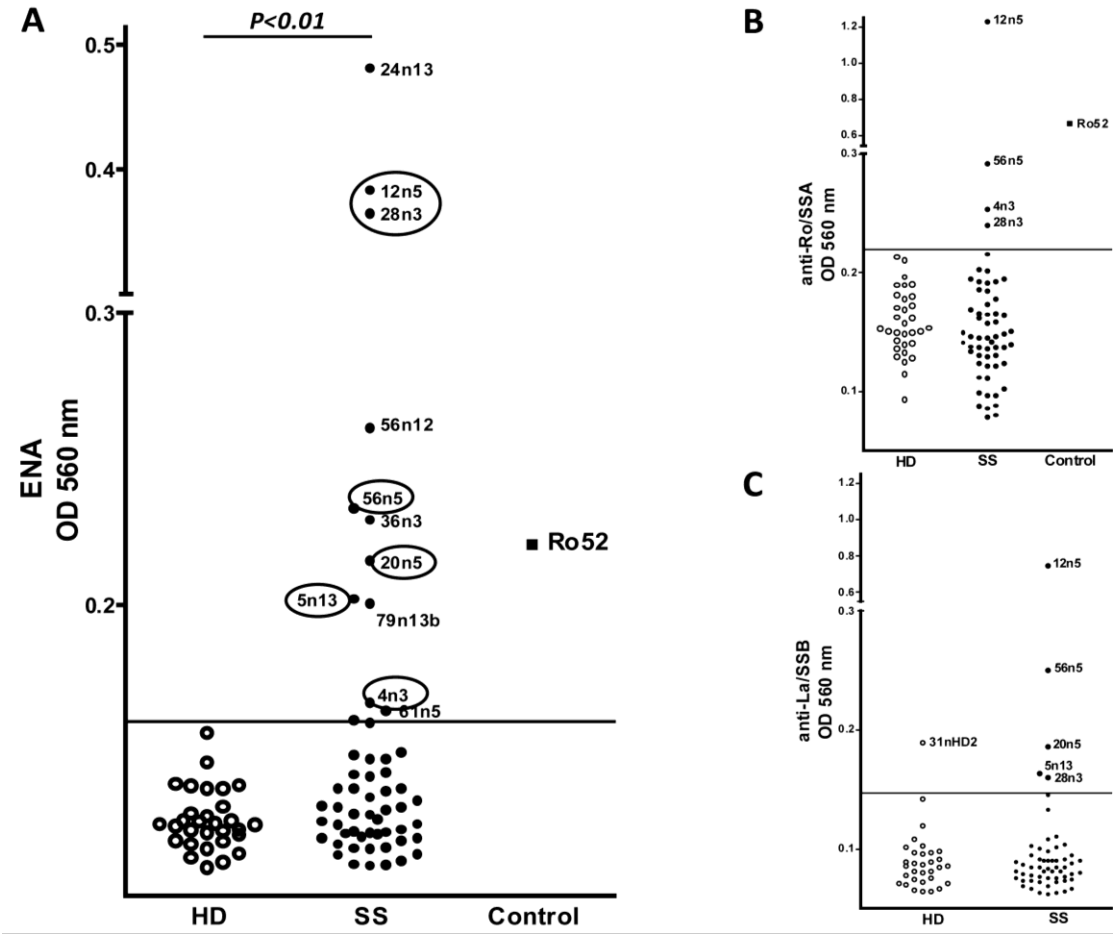

Figure 4.6 SS peripheral naïve B cells contain anti-Ro/SSA and anti-La/SSB clones.

(A) Naïve B cell antibodies from SS patients (n=56) and HD (n=31) were tested for reactivity against extractable nuclear antigens (ENAs) by ELISA. ENAs include a mixture of six purified antigens, including Ro/SSA and La/SSB. *P* value compares ENA+ versus ENA- clones from SS patients and HD. Naïve B cell antibodies from SS patients (*n=58*) and HD (n=32) were tested by ELISA specifically for reactivity against Ro/SSA **(B)** and La/SSB **(C)** antigens. Each graph shows the reactivity at a concentration of 10 μg/ml. Cut-off OD (560 nm) for positive reactivity is shown by the horizontal lines.

Chapter 5 | Results

Recombinant monoclonal antibodies from single memory B cells of patients with Sjögren's syndrome

5 | Analysis of memory switched and unswitched B cells antibodies cloned from peripheral blood B cells of Sjögren's syndrome patients.

5.1 Introduction.

As reported in Chapter 4, patients with SS are characterized by a significant reduction of circulating IgD-CD27+ switched and IgD+CD27+ unswitched memory B cells which could explain their accumulation in the target tissues (i.e., salivary glands) of these patients (**Figure 4.1**). Despite several evidences consistently support that altered B cell subpopulations are a feature of SS, further studies are needed in order to clarify whether these abnormalities represent primary pathogenic events or are secondary to the establishment of a chronic inflammatory process and whether they can predict disease evolution and response to treatment. To characterize the reactivity profile of circulating memory switched and unswitched B cells in patients with SS, I started to clone and express recombinant monoclonal antibodies from peripheral blood CD3-CD19+CD27+IgD- memory switched or CD3-CD19+CD27+IgD+ memory unswitched B cells of patients with SS. In this chapter, I will present data assessing the Ig gene usage (HC and LC) for both memory B cell subsets and data regarding the polyreactivity profile of the SS memory B cell monoclonal antibodies. Further evaluation of the reactivity profile of these antibodies is still under investigation and it will not be part of the current thesis.

5.2 Immunoglobulin (Ig) gene repertoire characterization of memory switched and unswitched antibodies cloned from SS peripheral blood B cells.

To characterize the Ig gene repertoire expressed by memory switched and unswitched B cells I studied seven patients with a diagnosis of primary SS (SS3, SS4, SS5, SS6, SS9, SS12 and SS13) (**Table 3.1**). CD3-CD19+IgD-CD27+ memory switched and CD3-CD19+IgD+CD27+ memory unswitched B cells were sorted as single cell by flow cytometry from peripheral blood of these patients. The analysis of 103 V(D)J gene segments from the memory switched B cells and 131 V(D)J gene segments from the memory unswitched B cells demonstrated a similar VH, D and JH gene repertoire in both memory B cell compartments (**Figure 5.1A**). Memory B cells from healthy controls are necessary to perform a better evaluation of the gene usage in SS patients. Thus, I performed a comparison with data previously published by the Wardemann's group. SS memory switched and unswitched B cells showed a similar V(D)J gene usage compared to controls [177]. Similar to the naïve B cells from SS patients (Chapter 4), VH3 was expressed more frequently (more than 50%) followed by VH4 and VH1 in both memory B cell compartments. I found a frequent usage of JH4 followed by JH6, JH5 and JH3 while JH1 and JH2 were expressed less frequently especially in the memory switched B cells. Igκ light chain usage (κ, 41 for memory switched and 74 for memory unswitched) was similar between the two memory B cell compartments (**Figure 5.2A**). Analysis of Vλ genes showed a reduced frequency of Vλ2 and a significantly increased of Vλ3 gene family usage in the memory switched B cells

compared to memory unswitched B cells which showed a higher frequency of Vλ distal genes (Vλ4 and Vλ6) (**Figure 5.2B**). Over expression of Jλ2/3 and a reduced frequency of Jλ7 was observed in both memory B cell compartments, as previously reported [168], thus suggesting a defect in receptor editing. Furthermore, no significant differences in CDR3 length and positive charges within the CDR3 were observed between memory switched and unswitched B cells (**Figure 5.1B**).

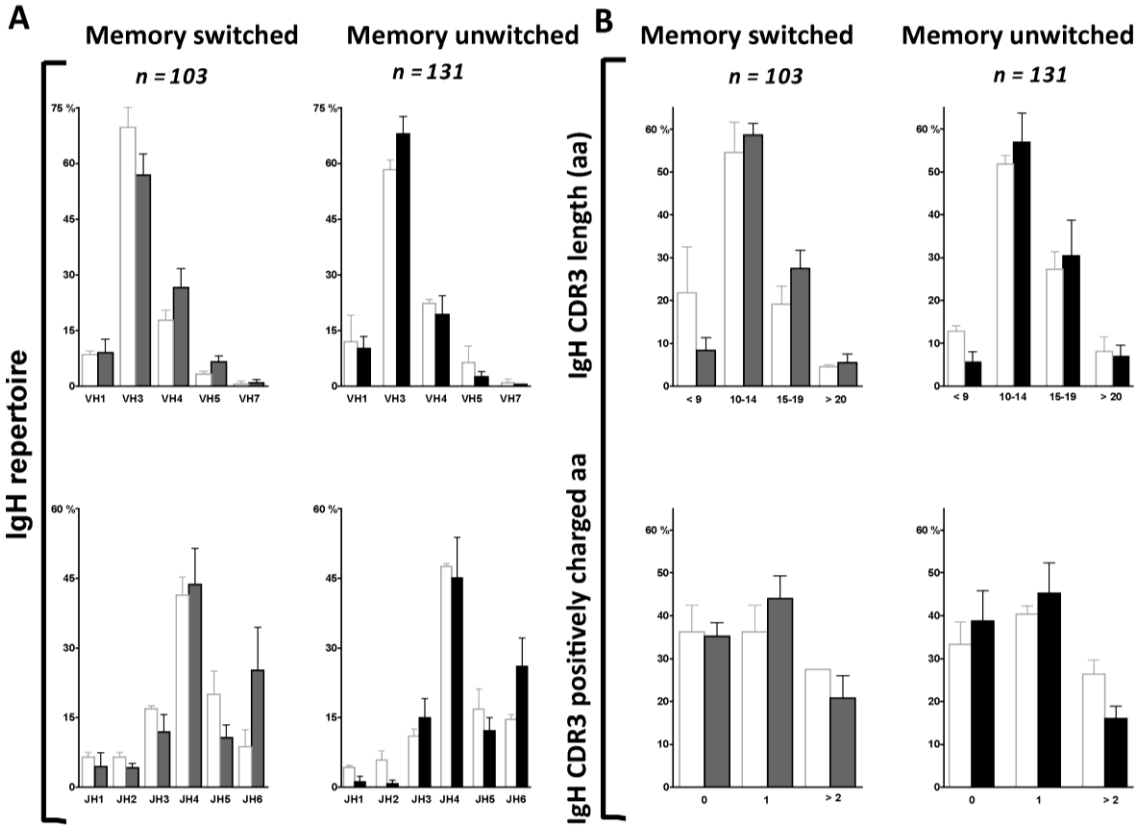

Figure 5.1 Ig heavy chain gene analysis of memory switched and unswitched B cells from SS patients.

Single memory switched and unswitched B cell antibodies from seven SS patients (memory switched) and six SS patients (memory unswitched) were analyzed for **(A)** IGH V family and J gene usage, and **(B)** IgH CDR3 aa length and positive charges. White bar graphs represent healthy controls, IgG+ (n=116) and IgM+ (n=113) for memory switched and unswitched, respectively [40, 184]. The absolute number of sequences analysed for SS patients is reported over each graph. Error bars in bar graphs indicate standard error of mean (SEM) for individual patient.

Mutational analysis is normally used to study whether a cell has experienced a positive antigen selection. Normally, a nucleotide substitution which brings to an amino acid replacement occurs less frequently in the FRs in order to maintain the right Ig fold, whereas is positively selected in the CDRs to increase the antigen affinity of the Ig. Thus, the comparison of the silent (S) to replacement (R) ratios in the FR and CDR regions is used to study the presence of antigen selection. Therefore, the numbers of S and replacement R mutations were calculated for each B cell clone within the FR regions 1, 2 and 3 and CDR1 and CDR2. CDR3 was excluded from mutational analysis since it is complicated to discriminate between point mutations and junctional variation as a result of recombination events in this region. As shown in **Figure 5.3**, R/S mutation ratios were calculated for the heavy chain (VH) and both light chains (Vκ and Vλ) in the FR and CDR regions separately. The graphs illustrate the R/S value for all the clones together. The R/S ratio of the CDRs of VH and both light chains genes from memory switched B cells was much higher in comparison with that in the FR regions, which is typical of an antigen-driven selection on their IGH genes (**Figure 5.3A**). Unswitched memory B cell antibodies showed a lower R/S ratios in the CDRs compared to memory switched B cell antibodies but still higher compared to FR regions, again suggesting an antigen-mediated selection in these B cell antibodies (**Figure 5.3B**). The R/S ratio of the CDRs of both light chains genes from memory unswitched B cells was lower in comparison with that in the FR regions probably due to the fact that these segments are longer, thus it is likely to have more random mutations occurring (**Figure 5.3B**).

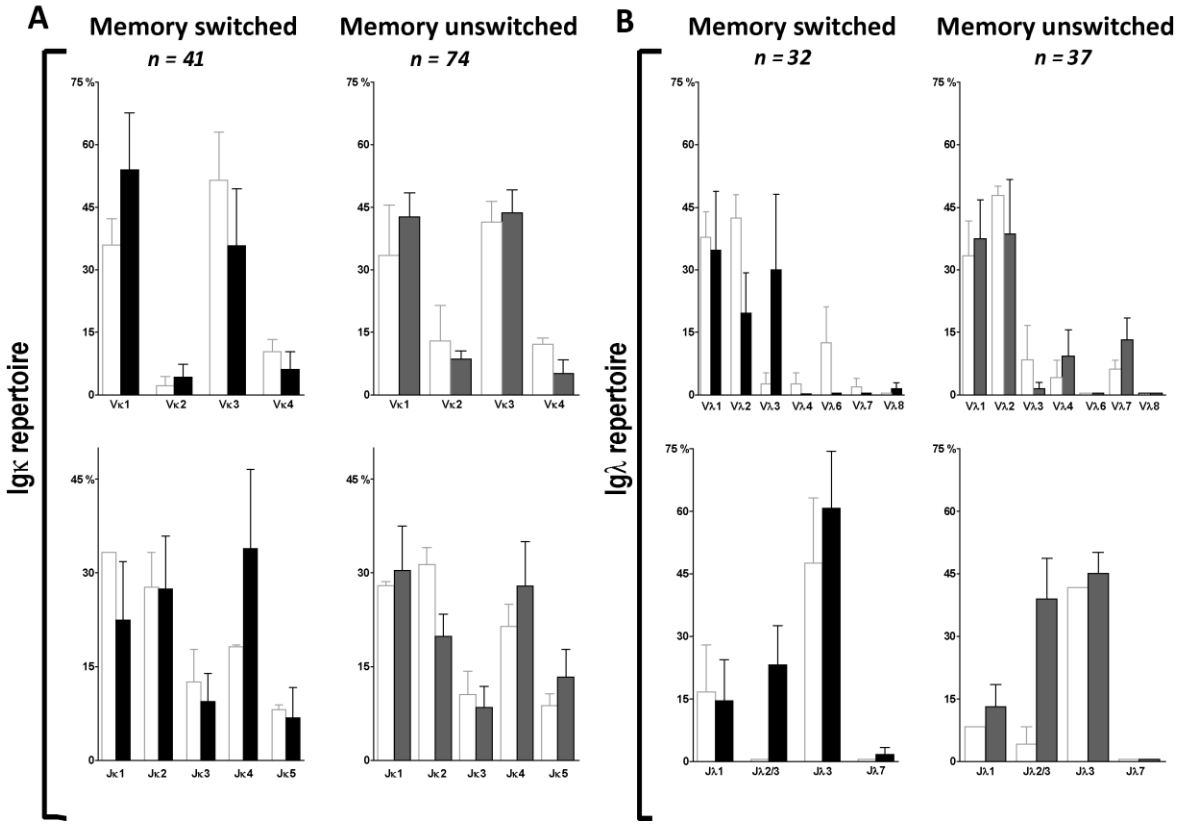

Figure 5.2 Ig light chain gene analysis of memory switched and unswitched B cells from SS patients.

Single memory switched and unswitched B cell antibodies from seven SS patients (memory switched) and six SS patients (memory unswitched) were analyzed for IGK **(A)** and IGL **(B)** V family and J gene usage. White bar graphs represent healthy controls, IgG+ (Igκ n=72; Igλ n=44) and IgM+ (Igκ n=72; Igλ n=36) for memory switched and unswitched, respectively [40, 184].The absolute number of sequences analysed for SS patients is reported over each graph. Error bars in bar graphs indicate standard error of mean (SEM) for individual patient.

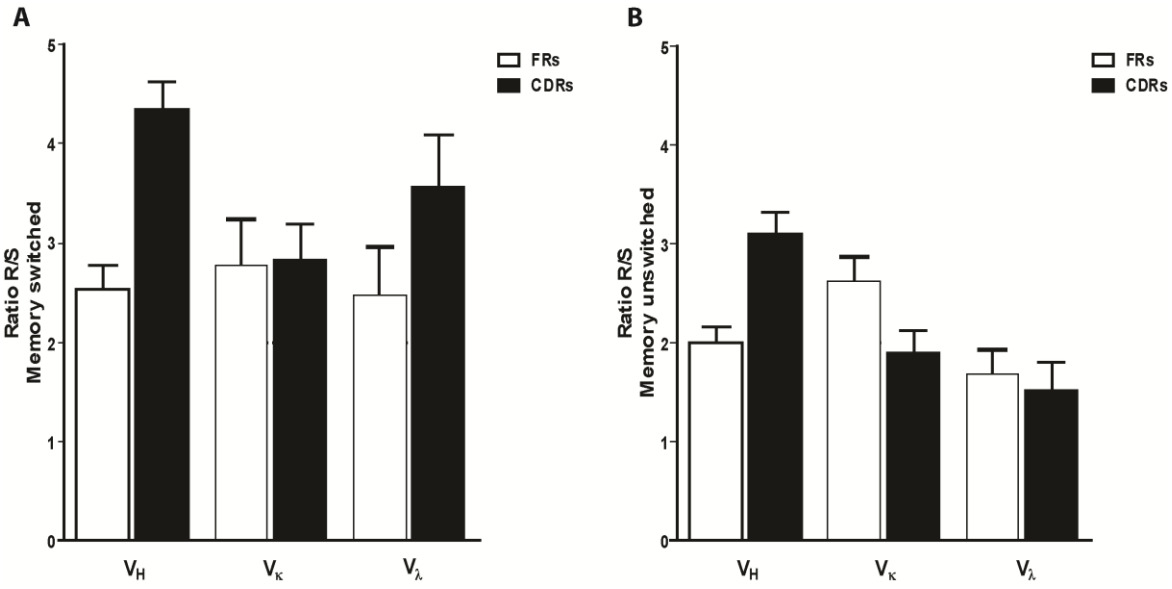

Figure 5.3 Analysis of replacement (R) and silent (S) mutations ratio in FR and CDR regions of memory switched and unswitched B cell antibodies.

Replacement:silent ratios (R/S ratios) were calculated for the complementarity determining regions (CDRs) (black) and framework regions (FRs) (white) for both heavy and light chains of memory switched (A) and unswitched (B) B cells. The R/S ratios for all the clones together are shown.

5.3 Polyreactivity profile of memory switched and unswitched B cell antibodies.

Out of all single memory switched and unswitched B cells isolated from SS patients, I cloned the matching IGH, IGK, and IGL chains genes of 16 memory switched and 32 memory unswitched B cells into specific expression vectors and produced the recombinant monoclonal antibodies *in vitro* (for full details of the repertoire and reactivity of the memory switched and unswitched B cell antibodies from SS patients see **Table 5.1** and **5.2**). I used only 39 out of 48 monoclonal antibodies to measure the polyreactivity by ELISA with structurally different antigens, as previously reported in Chapter 4 and [34], since 9 monoclonal antibodies had a concentration < 1 μg/ml. I observed, on average, 25 % of polyreactivity in the memory switched B cell antibodies and 11 % in the memory unswitched B cell antibodies. In details, 3 out of 12 memory switched and 3 out 27 memory unswitched B cell antibodies were reactive with at least two antigens in this ELISA, thus considered polyreactive (**Figure 5.4**). Normally, the frequency of polyreactive IgG and IgM memory B cell antibodies from healthy donors is around 22% and 1%, respectively. For instance, in SLE patients, it has been shown that the frequency of polyreactive IgG memory B cell antibodies is not significantly different from healthy donors. Therefore, based on the data published in the literature we can hypothesize that the frequency of polyreactivity in the memory switched B cell antibodies is not increased in SS patients compared to normal individuals [177]. Conversely, the frequency of polyreactive memory unswitched B cell antibodies seems to be increased compared to normal individuals.

These results are extremely interesting as circulating IgM memory B cells bearing a marginal zone-like phenotype [185] have long been implicated in the pathogenesis of SS [82].

Table 5.1 IgH and L gene repertoire analysis and polyreactivity of single memory switched B cells from SS patients. Clones highlighted in grey are those expressed as recombinant antibodies. (-) and (+), negative and positive charges within the heavy chain CDR3; length, number of amino acids within the CDR3; +, clones reactive in ELISA/IFA; -, clones non-reactive in ELISA/IFA; ND, not determined; C, cytoplasmic pattern; N, nuclear pattern.

Patient 3	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
10s.3	3-30*18	5-5*01	4*02	2	LPPSVDTHMAWSDY	1	14	κ	1-27*01	3*01	0	QSYNSALRGFT	1	11									
13s.3	3-9*01	6-13*01	1*01	2	APNSWAAEYFEH	1	12	λ	3-21*01	1*01	2	QVWDSSSDHYV	1	11	-	-	-	-	-	-	--	-	-
17s.3	3-15*04	3-10*01	3*02	4	DRDPYDSGSYGVFDM	1	15	κ	1D-39*01	2*01	1	QQSFSSPDT	0	9									
24s.3	4-30-4*01	2-2*03	6*02	1	WTVVVPPVSKPNFYYYAMDV	1	20																
25s.3	3-23*04	3-16*01	6*04	1	GPRASSLGRPGDV	2	13	λ	2-14*01	1*01	0	CSYTTRGTYV	1	10	+	-	-	-	+	+	C+N	-	-
27s.3	3-9*01	1-1*01	4*02	3	DAGGNWNDFPYHYFDS	1	16	λ	1-44*01	3*01	1	AACDGSLNGHVV	1	12									
28s.3	1-3*01	6-19*01	5*02	2	DLLSYIIVAGRGWFDP	1	16	λ	2-14*01	3*02	0	KSYTSTGSTPWI	1	12									
29s.3	4-39*01	6-19*01	1*01	0	QAVAGRVRYFQH	3	12	λ	8-61*01	7*01	0	VLYMGSGISV	0	10	+	+	-	+	+	+	N	+	+
31s.3	3-15*01	6-19*01	5*02	2	DGSSGRFTGWFDP	1	13	κ	4-1*01	1*01	1	QQYYSSPET	0	9									
33s.3	4-34*01	3-16*01	4*02	3	DGSLYEMGSYFDF	0	13	κ	1-5*03	2*01	1	QEHTSSSYT	1	9									
35s.3	4-39*02	4-17*01	4*02	5	HVRDYDDDFGYDY	2	13																
36s.3	3-30*17	6-19*01	4*02	1	GLSVTGTVATGFDY	0	14	κ	4-1*01	4*02	0	QQYSRIPLT	1	9									
37s.3	3-23*04	6-19*01	4*02	2	GEISNGWTYYSDY	0	13	κ	1-5*03	1*01	0	QQYNTHNT	1	8									
40s.3	5-a*01	3-10*01	5*02	0	HSTVSLVRGLSA	2	12	λ	1-44*01	3*02	2	LAWDDTLHGPV	1	11									
42s.3	4-b*02	3-3*02	4*02	1	LGFVGVVKIDS	1	11	λ	1-44*01	3*02	1	ATWNNDVLNGPWV	0	13									
43s.3	3-21*01	6-13*01	6*03	2	VAAQLDYYYMDV	0	12	κ	1-5*03	2*03	0	QQYNSYMYS	0	9									
45s.3	3-7*01	3-3*01	6*03	3	VYDFWSGSSANDNYYYYMDV	0	20	κ	1D-39*01	2*01	0	QQSFSTPYT	0	9									
46s.3	4-b*02	5-12*01	4*02	2	VTTMTDYFDF	0	10	κ	1-5*03	1*01	0	QQYNSFWT	0	8	-	-	-	-	-	-	--	-	-
54s.3	3-49*07	2-8*01	4*02	1	GPFTRIPPLKPVDY	2	14																
55s.3								κ	3-20*01	2*02	0	QQYGSSPQT	0	9									
								λ		3*01	1	GTWDSSLSSVV	0	11									
56s.3	4-59*02	5-24*01	4*02	1	QRWLQVFDN	1	9																
58s.3								κ	3-20*01	1*01	0	QQCGSFGT	0	8									
59s.3	4-39*01	6-19*01	4*02	1	GPVASTRYFDY	1	11																
60s.3	5-51*01	1-26*01	4*02	1	ANLGVLGRTYFDY	1	13	κ	3-11*01	2*01	1	QQCSDWLYT	0	9									
65s.3	3-48*01	4-23*01	3*01	4	EGNGELISDALDV	0	13	λ	2-23*02	3*02	0	YSYAGSSSFGNWV	0	13									
71s.3								κ	1-51*01	1*01	1	GTWDSSLSVGI	0	11									
72s.3	3-23*04	6-19*01	4*02	1	DLGSAWYMGS	0	10																
78s.3	3-21*01	2-8*02	4*03	1	GRGANYWSSFES	1	12	λ	1-44*01	3*02	2	AAWDDSLNAWV	0	11									
93s.3								κ	2D-40*01	2*01	1	VQRKEFPYT	2	9									

Patient 4	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
2s.4	1-2*02	3-3*02	5*02	2	GVMRPPRPEGWFDP	2	14	κ	1D-39*01	3*01	0	QQSYSMPRT	1	9	-	-	-	-	-	-	--	-	-
3s.4	3-23*04	3-22*01	4*02	2	DSHPFPSGQYYFDY	1	14	κ	1D-12*02	5*01	0	QQANSFPSIT	0	10	-	-	-	-	+	-	--	-	-
8s.4	3-48*01	5-5*01	6*02	2	EGAYGYNHGYQYYYGLDV	1	18																
33s.4	1-18*01	3-22*01	4*02	1	MSKSSGLYPYYFES	1	14	λ	2-14*01	1*01	0	SSYTSPSR	1	8									
34s.4	1-18*01	3-22*01	6*02	3	DLSYFDPAGPSFYYGMDV	0	18	λ	1-44*01	3*02	2	SVWDDSLNGWV	0	11									
37s.4	4-b*02	3-9*01	5*02	2	DLKGGRYYDLLARGNH	4	16	κ	1-5*03	2*04	0	QQYNSYPVQ	0	9									
40s.4	4-31*03	4-17*01	4*02	2	GVVYGGYADYCFDS	0	14																
42s.4	1-46*03	6-6*01	4*02	4	AQPSYSDSSDDFDY	0	14																
44s.4	4-34*01	3-9*01	5*02	0	GRRSNAYLRGASFGP	3	15																
49s.4	3-23*04	1-1*01	4*02	1	SGSNWNTYFDN	0	11	λ	2-11*01	1*01	0	SSYAGSYTAV	0	10	-	+	-	-	-	+	C	-	-
50s.4	3-74*01	5-24*01	4*02	3	DGGFYFDD	0	8																
54s.4	3-21*01	3-22*01	4*03	2	VLPVNTTDMGFDS	0	13																
62s.4	4-28*03	1-1*01	3*02	2	TSNHWNDAWAVDI	1	13	λ	1-44*01	1*01	2	AAWDDSLNGLYV	0	12	-	-	-	-	-	-	--	-	-
64s.4	3-33*03	4-4*01	4*02	3	DGNDHSNYYFDS	1	12	λ	2-8*01	1*01	0	SSYVGSNQYVF	0	11	-	-	-	-	-	-	--	-	-
73s.4	3-11*03	1-26*01	6*03	5	DNSEVGGTMDDFYYYMDV	0	18																
77s.4	3-48*01	6-13*01	4*02	2	DLPIAAADY	0	9																
78s.4	3-23*04	3-22*01	4*02	1	SSGTVYHHFDF	2	11																
80s.4	3-23*04	2-2*03	6*03	3	IAIGYDDYYMDV	0	12																
86s.4	3-23*04	3-9*01	5*02	1	GMTSWDP	0	7	λ	1-44*01	3*01	2	AAWDDSLNGVV	0	11									
89s.4	3-7*01	3-9*01	4*02	1	VGANWIIYYLDY	0	12	λ	2-8*01	3*02	0	SSYTGSYTLGV	0	11									
91s.4	3-48*02	2-2*03	3*02	4	GRRCDGSCYLPYDDALDI	2	18																

Patient 5	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
21s.5	3-30-3	3-10	3	2	YDTMVLWAGAFDM	0	13	κ	3-15	1	1	QHYENWWT	1	8									
26s.5	3-53	4-17	4	3	EFSPTVITRYCFDD	1	14																
35s.5	3-23	6-13	4	1	AWLGSSWMGYFDY	0	13	κ	4-1	1	0	QQYYSTSTWT	0	10	-	-	+	-	-	-	--	-	-
65s.5	4-59	2-21	4	1	RGVATVGAYYFDY	1	13	κ	1-17	3	1	LQDGNYPFS	0	9									
72s.5	3-72	1-7	4	3	AEKYSANYYAGDD	1	13	κ	1-16	5	1	QQFNDYPLT	0	9									
80s.5	3-9	2-21	6	1	GMGPCIIGACYAGYYNYYMDV	0	21	κ	3-20	4	0	QQYGSSSPLS	0	10									
84s.5	3-7	5-24	3	2	DLFLGGSNSRNVFDI	1	15	κ	4-1	4	0	QQYYSTPLT	0	9									
86s.5	3-30	3-3	4	3	DGDFWSGYCDY	0	11	κ	1-13	2	0	QQFNNYPHT	1	9									
Patient 6	**VH**	**D**	**JH**	**(-)**	**CDR3(aa)**	**(+)**	**Length**	**κ/λ**	**Vκ/Vλ**	**Jκ/Jλ**	**(-)**	**CDR3**	**(+)**	**Length**	**dsDNA**	**ssDNA**	**LPS**	**Insulin**	**ENA**	**Hep-2**	**Pattern**	**anti-Ro**	**anti-La**
5s.6	3-48	3-22	4	2	RGLYYDTSGSLKYFDF	2	16																
28s.6	3-15	6-13	4	1	AGMGSSRAEY	1	10	κ	3-20	4	0	QQYGSLPHT	1	9									
								λ	1-44	3	2	AAWDDSLMGV	0	10									
29s.6	1-2	2-15	5	1	VGIVVVLAATRGSYWFDP	1	18	λ	1-51	3	1	GTWDSSLSAVV	0	11									
37s.6	3-23	2-15	4	1	VGIVVVLAATRGSYWFDP	1	18																
76s.6	3-15	1-26	6	1	VGIVVVLAATRGSYWFDP	1	18	κ	3-20	2	0	QQYSSSSYT	0	9									
79s.6	3-15	6-6	4	1	DQFYSNFWYYFNY	0	13	κ	3-11	2	0	QQRSNWLPYT	1	10									
95s.6								κ	3-20	4	1	QQYDSSPFT	0	9									

Patient 9	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
6s.9	3-15	1-26	5	1	DGHLVGANV	1	9	λ	7-46	3	0	LLSYNNARV	1	9	-	-	-	-	-	+	C+N	-	-
20s.9	1-2	3-10	4	2	GYIGLGSYYGDCDY	0	14																
22s.9	4-31	3-10	4	3	LDGSGRHYDKGLDY	3	14																
23s.9	3-23	2-2	4	0	ASGVGCNSTSCYSAY	0	15	κ	3-20	4	0	QQYGSSPPLT	0	10									
24s.9	1-3	3-9	6	6	DPSTDRRYYDILTGYTEDYYGMDV	2	24																
30s.9	3-23	2-2	4	3	DDETAMLGVFQN	0	12	κ	1-39	1	0	QQSYSTGAWT	0	10	-	-	-	-	-	-	--	-	-
32s.9	3-15	6-19	3	2	ENASGSNLYAFDF	0	13																
33s.9	3-9	3-16	3	3	DMGSVIVPADAFDF	0	14																
34s.9	3-30-3	2-21	4	3	APDVDSAVVVGGSFDY	0	16																
39s.9	3-11	2-2	6	2	DRNIVPYYYYGMDV	1	14	λ	2-23	3	0	CSYAGSPV	0	8	-	-	-	-	-	-	--	-	-
47s.9	3-48	2-15	3	1	RRAIVVAGGALDV	2	13																
54s.9	3-74	5-24	6	1	DVGSPPGGIYSYALAV	0	16	λ	2-14	3	0	ISYRSSKTWV	2	10									
58s.9	4-28	4-17	6	1	VMRSTVTTSYYYYAMDV	1	17																
60s.9	3-30	2-15	3	2	EVDCSGGSCYSLAS	0	14	λ	2-23	3	0	CSYAGSPV	0	8									
81s.9	1-8	3-10	4	2	IGESWGSLFDH	1	11																
84s.9	5-51	3-16	3	1	QGAPNAFDV	0	9																
92s.9	3-11	2-2	6	2	IGPISASISYDFAMDV	0	16	κ	3-15	1	1	QHFDSWPRT	2	9									
94s.9	3-48	2-21	3	2	IGPISASISYDFAMDV	0	16																
95s.9	3-33	7-27	4	3	DSGTVGPSGDLDY	0	13																
96s.9	3-30	2-2	5	1	GQGYCSNINCYPFDP	0	15																

Patient 12	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
7s.12	1-69	3-22	4	2	QTSGIVVVQENFFDS	0	15																
9s.12	1-18	3-10	5	2	DHSLVRGAGDL	2	11	κ	1-39	2	0	QQSYTTPYT	0	9									
10s.12	4-59	3-10	6	2	VMYYYGSGNYLESYYYYGLEV	0	21	κ	1-27	3	0	QSYNSALRGFT	1	11									
20s.12	3-74	6-13	4	2	PAVSWEGFLDS	0	11	κ	3-20	2	1	QQYDSPPRT	1	9	-	-	-	-	+	-	--	+	+
34s.12	3-9	6-19	4	2	VAGSGWDYHFDL	1	12	κ	1-5	2	0	QYYNTFSAT	0	9									
35s.12	3-21	3-3	4	6	ENEEFDDFWSGFPRLYYFDY	1	20																
48s.12	3-7	6-6	6	2	DRQPSSYNGLDV	1	12	κ	2-28	2	0	MQGLQTPRT	1	9									
64s.12	4-39	3-10	6	2	QGADRRGSAYFFGMDV	2	16	λ	2-23	3	0	CSSASFTISWV	0	11	-	-	-	-	-	-	--	-	-
65s.12	3-9	3-3	4	2	DIRSGPGGLDS	1	11	λ	1-44	3	1	AACDGSLNGHVV	1	12									
68s.12								κ	3-20	1	0	QQFGSSPLWT	0	10									
78s.12	3-23	2-21	5	1	ESVAARGYFQF	1	11	λ	2-23	3	0	SSYAGNGTVA	0	10	-	+	-	+	-	+	N	-	-
79s.12	5-51	3-9	5	3	GGVVPDHDWFDQ	1	12																
89s.12	3-11	6-25	4	1	SLLSGTLSRVGYFDY	1	15	κ	3-15	1	0	QQYNQWPRT	1	9									
93s.12	4-61	2-21	2	3	AALGFTGGDYVDSSRYWYFDL	1	21																
94s.12	3-9	3-22	3	2	DMRTLSTMIVRGSDL	2	15																
23s.12	4-59	1-26	6	2	DQKIFSGSYSAYYYYGMDV	1	19	κ	1-39	4	0	QQTYGTPLT	0	9									
66s.12	3-23	4-23	6	2	GTTDMDV	0	7	κ	2-30	1	0	MQVSHWPRT	2	9									
67s.12	3-23	1-1	6	1	QQGFRVYYSALDV	1	13																
86s.12	1-46	1-1	4	1	GTDNSPTY	0	8																
90s.12	3-23	3-16	3	3	ESTVYMGNDAFDL	0	13	λ	1-40	3	1	QSYDTGLTGLKV	1	12									

Patient 13	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
11s.13								λ	2-14	3	0	SSYSTNGPLV	0	10									
17s.13	4-4	2-2	6	0	FSLGGHCSGTNCTPV	1	15																
23s.13	3-30	3-22	6	4	RGDYYDYYDSYYGMDV	1	16																
48s.13	4-4	6-19	3	1	ARTGWNLVNAFDL	1	13	λ	1-51	3	1	GVWDGSLSAVV	0	11									
59s.13	3-23	3-3	5	2	AWSSSVFGVDTLFDL	0	15	λ	1-47	3	2	AAWDDSLSGPV	0	11									
60s.13								κ	1-9	4	0	QQLNSHLT	1	8									
91s.13	3-30	4-17	6	2	QGLRDKTLNHYYIMDV	3	16	λ	1-40	3	1	QSYDSSLISWV	0	11									
96s.13								λ	1-36	3	2	AAWDDSLNGLGV	0	12									

Table 5.2 IgH and L gene repertoire analysis and polyreactivity of single memory unswitched B cells from SS patients. Clones highlighted in grey are those expressed as recombinant antibodies. (-) and (+), negative and positive charges within the heavy chain CDR3; length, number of amino acids within the CDR3; +, clones reactive in ELISA/IFA; -, clones non-reactive in ELISA/IFA; ND, not determined; C, cytoplasmic pattern; N, nuclear pattern.

Patient 3	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
1u.3	4-59	6-13	1	1	DTAYSSSWLPQRG	1	13	κ	1D-39	1	0	QQSYSTPRT	1	9									
2u.3								λ	4-69	3	1	QTWDTGIRV	1	9									
3u.3	4-b	6-13	4	1	VGRAAAGIPDY	1	11																
6u.3	3-30	2-8	2	2	DPGTDIGPGLFILVHV	1	16	κ	1-5	1	0	QQYKSYPGT	1	9									
7u.3	3-43	1-7	6	4	DAYDWKHGSDPYFSYVDI	2	18	λ	1-51	3	1	GTWDSGLSAGV	0	11	-	-	-	-	+	-	--	-	-
8u.3								κ	1-5	1	0	QQYKSYPGT	1	9									
9u.3								κ	2-30	2	0	MQGTHWPPYT	1	10									
13u.3	3-23	5-18	5	1	SHEWMQSLLRPHYLTPGP	3	18																
16u.3	3-74	5-24	4	4	GAGWDEDLATIQYFD	0	15																
19u.3	4-39	4-23	4	0	QVRYGTNPRFLTTG	2	14	κ	4-1	1	0	QQYYSTTQT	0	9									
20u.3								κ	4-1	1	0	QQYSGSPPSWT	0	11									
23u.3								κ	1-9	5	0	QQLNSYPIT	0	9									
25u.3	3-23	6-19	5	1	GHLYSSGWEWSTPG	1	14	κ	1D-39	1	0	QQSYSTLTWT	0	10									
26u.3								κ	1-16	1	0	LQYNIYPRT	1	9									
28u.3	3-48	3-22	4	3	DGHPNYYESSSSYYVPANYFDY	1	22	κ	3-15	4	0	QQYNNWPPFT	0	10									
32u.3	3-23	4-23	6	1	GAVVNNYYYYGMDV	0	14																
34u.3	5-a	4-23	4	3	HQEDYSYKSNSFDY	2	14	κ	1D-39	4	0	QQSYSAPLT	0	9									
35u.3	3-23	1-26	6	1	GLGNVGGTGGLETGC	0	15	κ	1-9	4	0	QQLNSYPLT	0	9									
37u.3								λ	1-47	3	2	AAWDDSLSGPV	0	11									
41u.3	3-7	2-2	4	0	PRPAAISRLTLV	2	12																
43u.3	3-23	3-9	3	4	DDSNFYDAFDI	0	11																
47u.3	4-59	4-17	6	2	GNYGDVCVDV	0	10	κ	1-5	1	0	QQYNSYSST	0	9									
48u.3								κ	3-15	3	0	QQYINWPQFT	0	10									
51u.3								κ	1D-39	5	0	QQSYSTSIS	0	9									
54u.3	3-21	5-24	4					κ	3-20	5	0	QQYGSSPIT	0	9									
59u.3	3-7	3-10	4	2	DSIFGFGELWGSVY	0	14	λ	1-40	3	1	QSYDSSLSAYWV	0	12									
60u.3	4-61	3-10	4	1	DKGSGSYYVLTTGP	1	14	κ	4-1	1	0	QQYFSSPT	0	8									
63u.3	3-48	3-3	6	3	DRGYDFWSGGGYMDV	1	15																
64u.3	3-7	6-13	4	3	DGTEAAGYYFDY	0	12	λ	4-69	3	1	QTWDTGIRV	1	9	-	-	-	-	-	-	--	-	-
65u.3	3-43	3-10	6	1	CKGMVRGVSGYYYYYYMDV	2	19	κ	3-20	5	0	QQYGSSPPIT	0	10									
68u.3	4-34	3-10	4	1	GPLTDYYGSGSLTTG	0	15																
70u.3	4-4	3-22	3	1	GGYDGSGYCLTLVT	0	14																
72u.3	3-30-3	6-19	4	1	GGTGYSSGWADY	0	12	κ	1-5	1	0	QQYNSYSWT	0	9									
73u.3	3-49	2-2	6	2	DGSHLVPAAQGYYYYYYMDV	1	20																
74u.3	3-20	3-9	2	0	TSYSNRGWTPATGYSITGPAA	1	21	κ	3-20	2	0	QQYGTSPNT	0	9									
75u.3	4-34	2-2	5	2	SMSCDSSSCKGRGWFDR	3	17	κ	4-1*01	1	0	QQYYSPPPWT	0	10	-	-	-	-	+	-	--	-	-
76u.3	3-74	3-10	4	1	ALGFYGSGSYSGDY	0	14	λ	2-14	3	0	NSYTTSSTLV	0	10	-	-	-	-	-	-	--	-	-
78u.3								κ	3-20	5	0	QQYHSSPVT	1	9									
79u.3	3-30	2-21	3	2	PAGVVVTDDAINI	0	13																
80u.3								κ	3-11	4	0	QQRSNWSLT	1	9									
85u.3	4-61	2-8	5	2	SPPTNGGEWFDP	0	12																
86u.3	5-51	2-2	4	0	RYCLSASCYTPLTTG	1	15																
87u.3	3-11*01	2-2*03	3	2	DRGCSSPSCYGGAFDL	1	16	λ	4-69	3	1	QTWDTGIQV	0	9	-	-	-	-	-	-	--	-	-
88u.3	3-23	4-11	6	2	GGNDYNNAYYYYYYMDV	0	17	λ	2-8	3	0	ASYTGSNNFV	0	10									
93u.3	3-23	6-13	4	0	NRASSSWYYLTTG	1	13																
94u.3								κ	2D-28	4	0	MQTLQIPLT	0	9									

Patient 4	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
1u.4	4-59	3-16	6	1	QNSHNYYYYMDV	1	12																
3u.4								λ	1-40	3	1	QSYDSSLSGSV	0	11									
4u.4	5-51	1-7	6	4	LKGENWNYHYYGMDV	5	16	λ	2-23	3	0	CSHAGSSLSL	1	10	-	-	-	-	-	-	--	-	-
11u.4	3-64	3-22	3	3	SWDTSGNPHDVYDI	1	14																
13u.4	3-23	3-3	6	2	DRGGVRIFGGYYYGMDV	2	17	κ	3-11	5	0	QQRSNWPIT	1	9	-	-	-	-	-	-	--	-	-
19u.4	3-23	6-6	6	2	NGDHSSSSSMDV	1	12	κ	3-15	1	0	QQYNNWPPWT	0	10	-	-	-	-	-	-	--	-	-
22u.4	1-46	6-19	6	2	DRRYSSVYGMDV	2	12	κ	2-D28	3	0	MQALQTPFT	0	9	-	-	-	-	-	-	--	-	-
25u.4	3-21	3-10	6	2	DSPPGGPGSGSSLTTTGMDV	0	20																
30u.4	3-21	6-19	6	2	PGGYSSGWYKDFYYYMDV	1	18																
33u.4	3-7	3-10	3	3	GDYHSGTFIDAFDI	1	14																
34u.4	3-23	2-2	1	2	DLWSCSSSSCDVGTCY	0	16																
40u.4	3-72	5-12	4	1	GYVGAAQSNFDY	0	12	κ	1-5	5	0	QQYNSYPITF	0	10									
41u.4								κ	3-20	2	0	QHYGSSRWT	2	9									
42u.4	4-61	3-22	2	2	AVYESSGYQGIYWYSDL	0	17																
46u.4	3-23	6-19	4	1	DGVGSSGWYYLATG	0	14																
50u.4	3-72	2-21	6	5	AYCDDDCYSDFYYYMDV	0	17	λ	1-51	1	1	GTWDSSLIAYV	0	11									
52u.4	3-23	2-2	6	3	DNAGYRGADYYYYMDV	1	16	λ	1-44	3	2	AAWDDSLNGPV	0	11									
53u.4								λ	2-11	1	0	FSYAGSYTYV	0	10									
61u.4								κ	3-11	4	0	QQRSNWPQT	1	9									
								λ	7-46	3	0	FLFYSGAWV	0	9									
65u.4	3-23	2-2	6	2	RDCSSTSCYLVTLDH	2	15	κ	1-9	4	0	QQVNSYPPT	0	9									
67u.4	3-30	2-2	6	1	GRVPAAIYYYMDV	1	13																
75u.4	3-15	2-8	6	2	GADYYYYYMDV	0	11																
76u.4	3-33	2-2	6	2	EYQMVKAGYMDVW	1	13	κ	3-15	2	0	QQNNWPRT	1	8									

Patient 5	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
11u.5	Apr-59	3-10	6	2	LGPDSVYHYYYMDV	1	14	κ	2-40	3	0	MQSLQTFTF	0	9	-	-	-	-	-	-	--	-	-
24u.5	3-23	6-19	4	1	VGGFGPPLDN	0	10	κ	3-11	4	0	QQRANWPLT	1	9									
29u.5	5-51	6-19	4	1	LGGAVTLHYFDY	1	12	λ	1-44	3	2	AAWDDSLMGV	0	10	-	-	-	-	-	+	N	-	-
32u.5	3-73	2-8	1	1	LMGSPEYFGH	1	10	κ	1-16	4	0	QQYNRHPLT	2	9									
36u.5	4-34	6-13	5	2	DGNLTAAATRWFDP	1	14	κ	1-8	1	0	QQYYSYPRT	1	9	-	-	-	-	-	-	--	-	-
71u.5	4-61	6-19	6	1	ALPPYYYYYGMDV	0	13	κ	1-51	3	1	GTWDSSLSAGV	0	11									
76u.5	3-7	3-16	4	1	MPFDY	0	5	κ	1-17	4	0	LQHNSYPLT	1	9									
77u.5								λ	7-43	3	0	LLYYGGVRV	1	9									
80u.5	4-30-2	3-10	4	2	DGSGSSLPFDY	0	11	κ	3-20	1	0	QQYGTSPWT	0	9	-	-	-	-	-	-	--	-	-
81u.5	4-34	3-3	4	2	KHWSTDVTTNLDY	2	13																
84u.5	4-4	3-22	1	4	DLYYYDSSGYYLYYFQH	4	18	κ	3-20	2	0	QQYGSSPYT	0	9	-	-	-	-	-	+	N	-	-
96u.5	3-21	3-16	5	1	GTLTSGGGPYNWFDP	0	15																

Patient 6	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
11u.6	1-46	3-9	5	3	DEGSTGYSRLDP	1	12	κ	1-39	2	0	QQSYRYPYT	1	9									
12u.6	3-48	6-25	6	3	DLRDGYGMDV	1	10	κ	3-20	4	0	QQSGSSLT	0	8									
19u.6	3-53	1-26	2	2	ARDSGSYYVHWYFDL	2	15																
23u.6	4-34	7-27	4	0	GRLGGAAN	1	8	κ	1-27	1	0	QNYNSAPRT	1	9									
24u.6	4-39	5-5	6	1	QYGMDV	0	6	λ	2-14	3	0	SSYTNSNTFWV	0	11									
31u.6	4-4	3-22	4	1	GISSGWAGKFDY	1	12																
39u.6								λ	2-23	3	0	SSYAGGKTWV	1	10									
41u.6	3-48	2-15	4	2	GGYCSGDSCYGGYYFDY	0	17																
42u.6	4-31	4-17	3	4	REGDPDAFDI	1	10																
48u.6	3-7	4-17	6	1	PIYGSSSGLSGMDV	0	14	κ	1-39	1	0	QQSYSSLWT	0	9									
51u.6	3-21	3-10	3	1	VTGNPGAFDI	0	10	λ	2-11	3	0	CSYTGSYTSV	0	10									
55u.6								κ	1-39	2	0	QQSYNTPYT	0	9									
67u.6	7-4-1	3-22	3	2	TYYDSSGYYYVFDI	0	14	κ	3-20	2	0	QQHGSSPYT	1	9	-	-	-	-	-	-	--	-	-
69u.6	3-72	1-7	3	1	GGITGPRITFDI	1	12	κ	1-5	4	0	QHYNSYPLT	1	9	-	-	-	-	+	-	--	-	-
74u.6	3-23	2-2	6	1	CGLAGSSTSCYSYGMDV	0	17																
75u.6	3-23	3-3	3	2	LSWGEPNGDFFGI	0	13	κ	1-17	4	0	LQHNSYPLT	1	9	-	-	-	-	-	-	--	-	-
77u.6	4-39	5-5	4	2	SGFFYDSDGYPMYNFHY	1	17	κ	3-11	4	2	QQRSNWPL	4	9									
81u.6	1-2	4-23	4	3	VRDSIGNSELDF	1	12	κ	1-5	4	1	QQYDTFPLT	0	9	-	-	-	-	+	+	C	+	+
83u.6								λ	7-43	1	0	LLYYGGGYV	0	9									
87u.6	3-21	6-19	6	2	LTVAGNHDYYFYGVDV	1	16	κ	2-28	4	0	MQAKQAPPT	1	9									
89u.6	3-7	3-10	4	1	VHNYYGSGSYSSYYFDY	1	17	λ	2-14	3	0	SSYTSSSTLGV	0	11	-	-	-	-	-	+	C+N	-	-

Patient 12	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
1u.12	1-3	6-19	4	2	DLAVAGSQFDS	0	11																
4u.12	4-34	3-10	4	1	GRGVALRGPNYFDY	2	14																
5u.12	3-30	3-22	6	2	ASVSGFSSGYYGDYYYGMDV	0	20																
9u.12	1-18	3-22	4	3	DAYYYDSSGYYYPHYFDY	1	18																
26u.12	4-4	6-6	4	0	PGILRPH	2	7																
28u.12	3-7	???	5	2	EPWFDP	0	6	κ	3-20	5	1	QQYDRKT	2	7	-	-	-	-	-	-	--	-	-
29u.12	3-11	3-10	2	2	DANYYGSGIIGWYFDL	0	16	κ	1-39	4	0	QQSYSTPLT	0	9									
31u.12	3-7	6-6	3	3	HRQWAAARPDDAFDI	3	15																
32u.12	3-11	3-3	6	1	LGFWGSYGMDV	0	11	λ	2-8	1	0	SSYAGSNNYV	0	10	-	-	-	-	-	-	--	-	-
40u.12	3-23	6-19	4	1	GRASGWTSFDY	1	11																
45u.12	3-23	3-22	5	3	AGRYNDYDIWFDP	1	13																
46u.12	4-59	4-23	3	3	HDYGGNAHADSFDI	2	14																
47u.12	4-39	1-7	4	2	HALGGNYDFDY	1	11	κ	3-20	5	0	QQYGT	0	5									
52u.12	3-23	4-17	4	3	DTGDYGNSFDY	0	11																
59u.12	5-51	1-1	5	0	HRAPAGTFQPFHL	3	13	κ	3-20	3	0	QQYGSSPPKT	1	10									
62u.12	4-59	3-16	4	2	RYYGLGEYHDY	2	11	λ	2-11	3	0	CSYAGSYTSFVV	0	12									
65u.12	1-46	3-10	6	2	DPMVRGLSYHGMDV	2	14																
69u.12	3-23	3-9	4	2	GRSGRRGYFDWLGIDY	3	16	λ	6-57	3	2	QSYDNDNLWV	0	10									
75u.12	3-30	6-19	4	2	GREWLHPFDY	2	10																
77u.12	4-39	2-8	5	0	PYKASGWWQGGHF	2	13	κ	1-9	3	0	QQLNSYPPFT	0	10									
80u.12	3-23	2-8	4	2	GHCANGVCYDSVDY	1	14	κ	1-9	2	0	QQLNTYPRT	1	9									
81u.12	1-18	5-5	4	2	KESHQLLVFDY	2	11	λ	2-14	3	0	SSYTTINTL	0	9									
86u.12	4-59	3-16	4	1	AGGGTPFDY	0	9	λ	1-44	3	3	AAWDDSLNGHWE	1	12									
87u.12	5-51	3-3	4	2	PRREWEPPRY	3	10	κ	1-39	1	0	QQSYSAPVT	0	9									
91u.12	5-51	3-9	4	1	GSGYFDY	0	7	κ	3-20	1	0	QQFGSSPRT	1	9									
92u.12	4-59	6-25	4	2	AGGIQSEQGIFDH	1	13	κ	1-5	2	0	QQYNSYSYT	0	9									
93u.12	4-59	4-4	4	2	YGVDYFDY	0	8	κ	3-15	2	1	QQYNNGPPDYT	0	11	-	-	-	-	+	+	N	-	-

Patient 13	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length	dsDNA	ssDNA	LPS	Insulin	ENA	Hep-2	Pattern	anti-Ro	anti-La
6u.13								κ	1-5	1	0	LQYNGNTWT	0	9									
7u.13	1-24	?	5	1	LRARAWFDP	2	9	λ	2-14	3	1	SSYRSNNTLDVV	1	12	-	-	-	-	+	-	--	-	-
8u.13	1-2	4-17	6	4	ESDYDPIMDV	0	10	κ	4-1	4	0	QQYYSTPLT	0	9	-	-	+	-	-	-	--	-	-
								λ	2-14	3	0	CSYSSSSTSVV	0	11									
9u.13	3-30	4-17	5	3	VNDYGDYGWFDP	0	12	κ	3-11	5	0	QQRSNWPPIT	1	10									
10u.13	1-18	3-22	3	2	TLTSRITMIVVVIDAFDI	1	18	λ	1-47	3	2	AAWDDSLSGPV	0	11									
11u.13	4-39	3-22	2	1	LYFSMTVALRYFDL	1	14	λ	3-9	3	1	QVWDSSIGV	0	9	-	-	-	-	-	-	--	-	-
21u.13	1-18	3-22	4	3	DRGAYYYDSSGYYVGFDY	1	18	λ	7-43	3	0	LLYYGGAQGV	0	10									
34u.13	3-9	5-24	4	2	DIGYNYGLRGFDS	1	13																
46u.13	5-51	3-22	5	2	HNKNYYDRSQLGFDP	3	15	λ	1-44	3	2	AAWDDSLNGPV	0	11									
47u.13	3-30	3-10	4	3	DYVGDNSFFDH	1	11	λ	4-69	1	1	QTWDTGIQV	0	9	-	-	-	-	-	-	--	-	-
48u.13	1-2	2-2	4	2	LEVVPAAMNDY	0	11	κ	3-11	1	0	QQRSNWPPWT	1	10									
56u.13	5-51	4-23	4	1	RGGSGSNVYFDY	1	12	λ	1-51	3	1	GTWDSSLSAVV	0	11	-	-	-	-	-	-	--	-	-
57u.13	4-59	?	3	3	DGADAFDI	0	8	κ	4-1	1	0	QQYYSTPWT	0	9	+	+	+	+	+	-	--	+	+
58u.13	1-2	3-16	4	3	PIWGDDVEGY	0	10	λ	4-60	3	3	ETWTSKEEI	1	9									
59u.13	3-9	3-3	4	2	DAQLSGFNYFDS	0	12	κ	1-5	1	0	QQYHFYST	1	8									
60u.13	3-11	3-10	4	1	SKGDY	1	5	κ	3-11	4	0	QQRSNWPLT	1	9									
68u.13	3-30	6-19	4	2	DLAPGFTSAFDY	0	12	κ	3-15	1	0	QHYNNWPPWT	1	10									
71u.13	3-48	1-26	5	2	DRELVGATKFNP	2	12																
72u.13	3-30	3-10	4	2	DLAPGFTSAFDY	0	12	κ	3-15	1	0	QHYNNWPPWT	1	10	-	-	-	-	+	-	--	-	-
79u.13	3-30	6-13	6	1	SQVAAAGRYGMDV	1	13	λ	1-40	3	1	QSYDSSLSGWV	0	11									
81u.13	4-34	2-15	4	1	GPRSSPIVVVVAVDY	1	15	κ	3-11	5	0	QQRSNWPPVT	1	10									
								λ	1-51	3	1	GTWDSSLSAV	0	10									
82u.13	4-59	5-5	4	1	ALGYNYGYRYFDY	1	13																
83u.13	3-7	3-3	6	3	DVIFEVLRFYYYGMDV	1	16	κ	1-17	1	1	LQDYNYPRT	1	9									
92u.13	3-7	5-24	3	2	LSRDGYNYNAFDI	1	13	κ	1-39	1	0	QQTYSAPGRSAK	2	12	+	+	+	+	+	-	--	-	-
93u.13	3-23	5-5	3	1	GIQLWPGGAFDI	0	12	κ	2-28	2	0	MQALQTPYS	0	9	-	-	-	-	-	+	N	-	-
94u.13	3-9	6-19	4	2	DSRRKSIAVAGAYFDY	3	16	κ	1-12	2	0	QQANSFPYS	0	9	-	+	-	-	-	+	N	-	-
96u.13	3-30	6-19	4	2	DLAPGFTSAFDY	0	12	κ	3-15	1	0	QHYNNWPPWT	1	10									

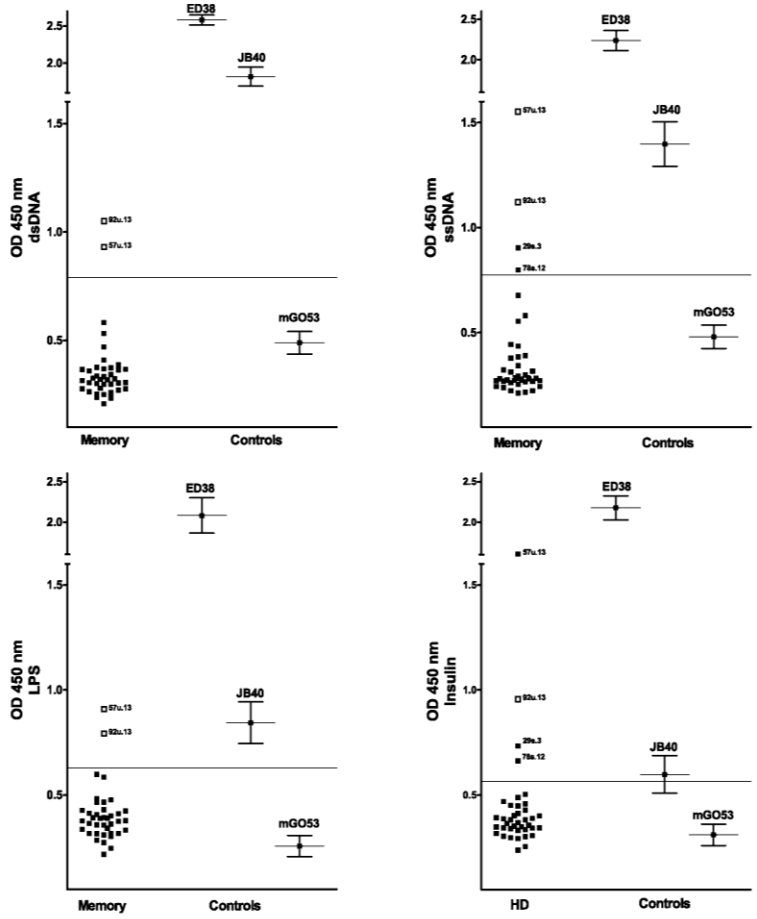

Figure 5.4 Polyreactivity of memory B cell antibodies from SS patients and HD.

Memory B cell antibodies from SS patients (*n=39*) were tested for reactivity with dsDNA (top left), ssDNA (top right), LPS (bottom left) and insulin (bottom right) by ELISA. Each graph shows the reactivity at a concentration of 1 μg/ml. The cut-off OD (450 nm) at which antibodies were considered reactive is shown by the horizontal lines and it shows the result of two independent experiments. Data points represent individual antibodies. Internal controls for polyreactivity (empty dots) are shown in each graph and include mGO53 (negative; [34]), JB40 (low polyreactive; [34]), and

ED38 (highly polyreactive; [34]). Error bars indicate standard error of mean (SEM) for the internal controls.

Chapter 6 | Results

Generation and characterization of recombinant monoclonal antibodies from single B cell isolated from the rheumatoid arthritis synovium

6 | Characterization of synovial B cell monoclonal antibodies.

6.1 Introduction.

As previously reported in Chapter 1.3, a subset of RA patients can develop ectopic lymphoid structures in the synovium which represent functional niches of autoreactive B cells contributing to autoimmunity independently from SLO. However, whether antibody production within RA synovium is driven by the same antigen(s) as in SLO is poorly understood. Moreover, previous studies have shown that local B cell activation and differentiation of B cells into antibody-producing plasma cells may occur directly in RA synovial tissue [163]. In the present study I analysed the V gene usage, mutational frequency and clonal diversification of RA synovial single-sorted B cells. In particular, clonal relationship analysis has been done in order to support the idea that in RA synovial tissue a continuous activation and differentiation of specific B cell clones may occur as a result of a local antigen-driven process. The novelty of my work is the possibility of investigating the nature of such putative dominant antigen(s) by identifying the immunoreactivity of synovial-derived monoclonal antibodies. For the purpose of the thesis, I will present data on the reactivity of synovial B cells towards citrullinated antigens.

6.2 RA patients.

Out of 96 single CD19+ B cell sorted from each of the 2 out of 4 RA patients used, I obtained successful sequences from a total of 139 different VH and JH regions (RA1-3=83, RA2=56), 94 Vκ and Jκ regions (RA1-3=67, RA2=27), and 81 Vλ and Jλ regions

(RA1-3=53, RA2=28). Clones with matching and productive VH and VL products were used for downstream cloning and recombinant antibody expression. All productive VH and VL products were analysed for the presence of V gene somatic mutations.

6.3 Immunohistological characterization of synovial tissue of patients with RA.

Synovial tissue was obtained from knee joint replacement surgery from 4 patients with established RA (**Table 3.1**). All samples were assessed by immunohistological staining in order to define the stage of lymphocytic aggregation, i.e. the presence of B (CD20) and T (CD3) cells, plasma cells (CD138) and macrophages (CD68). The sample RA1 was classified as grade 3 which defines larger aggregates with often T/B cell compartmentalization and the presence of FDC network (**Figure 6.1A and 6.1B**). The second sample RA2 showed mainly plasma cells infiltration (**Figure 6.1C**). Sample RA4 displayed larger aggregates with T/B compartmentalization and the presence of FDC network, thus classified as grade 3. Sample RA5 displayed few lymphocytes infiltration, thus classified as diffuse (data not shown). For the latter two samples, single CD19+ synovial B cells were sorted but the generation of recombinant antibodies and their analysis is still under way.

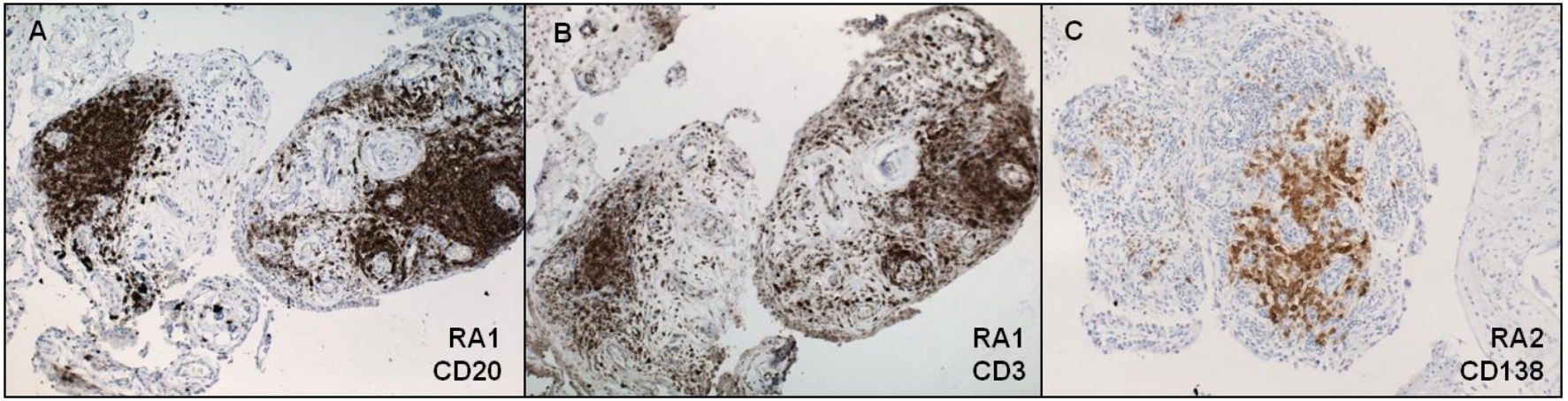

Figure 6.1 Representative immunohistological characterization of synovial tissue sample from patients with RA.

In order to assess the presence of B cells, T cells and plasma cells paraffin tissue sections were stained for CD20 (A), CD3 (B) and CD138 (C), respectively.

6.4 V gene repertoire analysis of synovial B cells.

Single CD19+ B cells were sorted from synovial cell suspensions and the V gene repertoire was determined (**Figure 6.2A**). Analysis of the B cell heavy chain isotype showed that 33% of the synovial B cells expressed IgM, while 40% expressed IgG, and 27% expressed IgA (**Figure 6.2B**). A sequence analysis of the synovial B cells showed that the VH gene repertoire was not significantly different when compared to peripheral blood CD5-IgM+ B cells in healthy donors (**Figure 6.3A**). As previously published by the Berek's group [163, 172], CD5-IgM+ B cells denote naïve B cells characterised by V genes in an unmutated germline configuration and they were used as controls in previous publications [163]. In the IgM+ and IgA+ populations an overrepresentation of VH1 rearrangements was observed but it was not statistical significant. Statistical significant differences were found mainly in the JH gene repertoire of synovial B cells when compared to controls. Both JH2 and JH3 were overrepresented in the IgM synovial B cells. In the IgG synovial B cells JH4 was found less frequently and JH5 was overrepresented compared to controls. The CDR3 length analysis showed that the IgM+ synovial B cells were characterised by significantly longer CDR3 compared to IgG and IgA, as expected since 40% of the IgM V genes were in an unmutated germline configuration (**Figure 6.3B, top panel**). Significant differences in the CDR3 positive charges were observed in the IgA+ and IgG+ synovial B cells with more positive charges compared to IgM+ synovial B cells (**Figure 6.3B, bottom panel**).

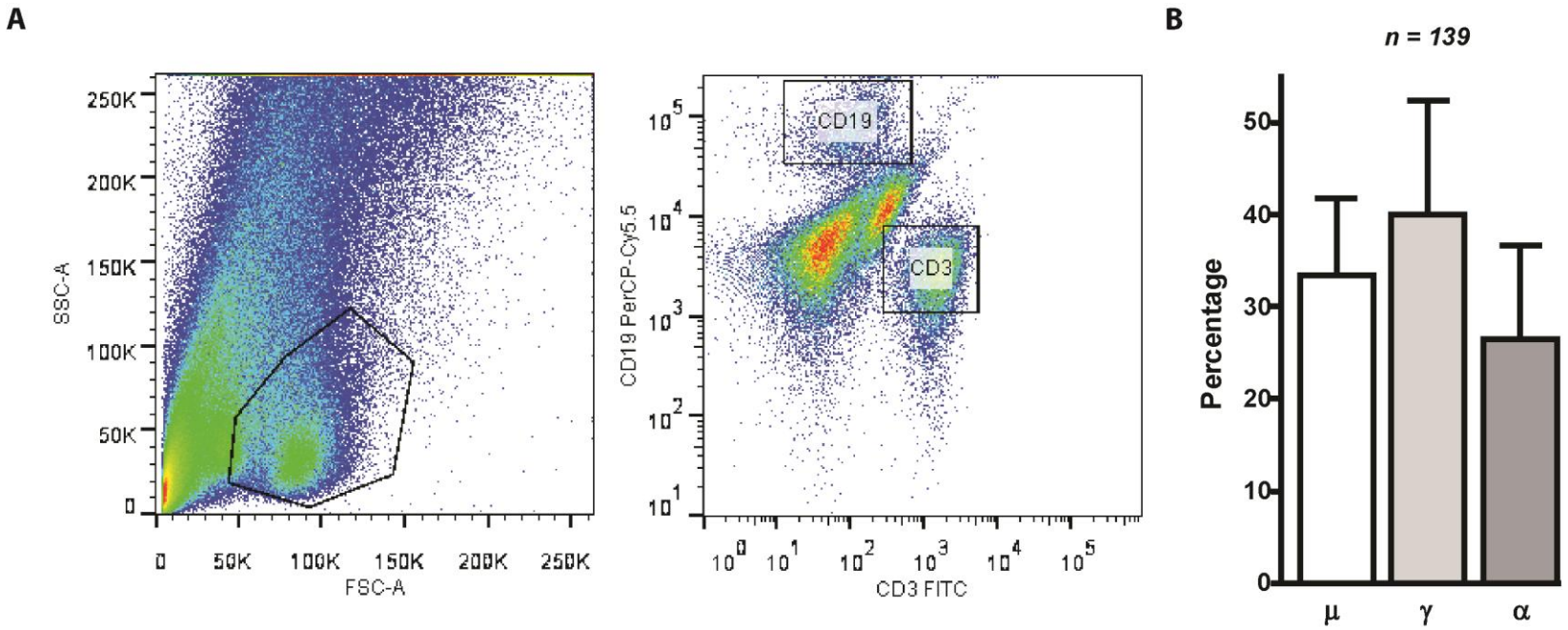

Figure 6.2 Isolation strategy of single CD19+ synovial B cells and comparison of the frequencies of μ, γ, and α heavy chain isotype.

(A) Mononuclear cells from RA patients were surface labelled with fluorochrome-coupled anti-CD19 and anti-CD3. The sorting gate strategy for single CD19+CD3- is shown. **(B)** The frequencies of μ, γ, and α heavy chain isotype among all CD19+ B cells are shown.

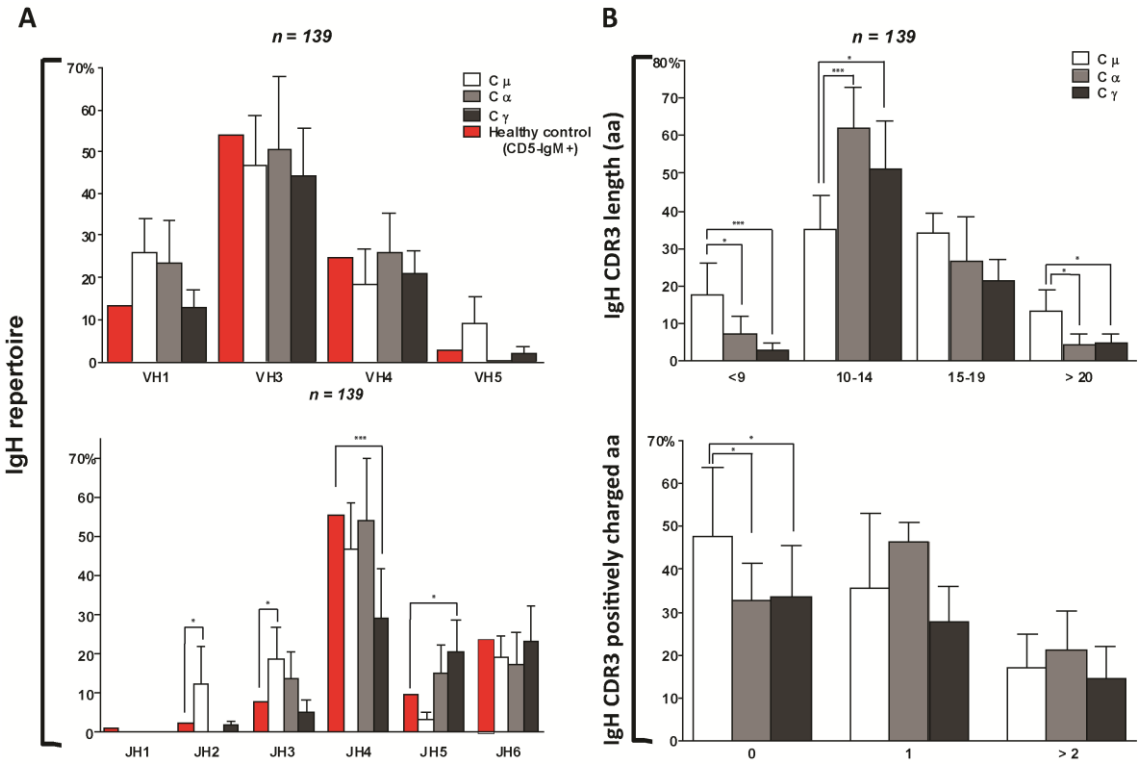

Figure 6.3 IgH gene analysis of single CD19+ synovial B cells from RA patients.

(A) The VH and JH gene repertoire of single CD19+ synovial B cells for each individual heavy chain isotype, μ (white), α (grey), and γ (black) is shown. The red bars indicate the VH and JH gene repertoire of peripheral blood naïve B cells [172]. **(B)** IgH CDR3 aa length and positive charges is shown for each heavy chain isotype separately. The absolute number of sequences analysed is reported over each graph. Error bars in bar graph indicate standard error of mean (SEM) for individual patients. *P* values compare data from RA synovial B cells to peripheral blood naïve B cells or from RA synovial B cells with different heavy chain isotype and it was calculated using the chi-square test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Similarly, no significant changes in V and J gene usage for kappa and lambda chains were observed between the synovial B cells and CD5-IgM+ B cells used as control (**Figure 6.4**). Only a trend towards an increase in Vλ1 and Jλ1 gene usage was observed in the synovial B cells (not statistically significant) suggesting a possible defect in receptor editing.

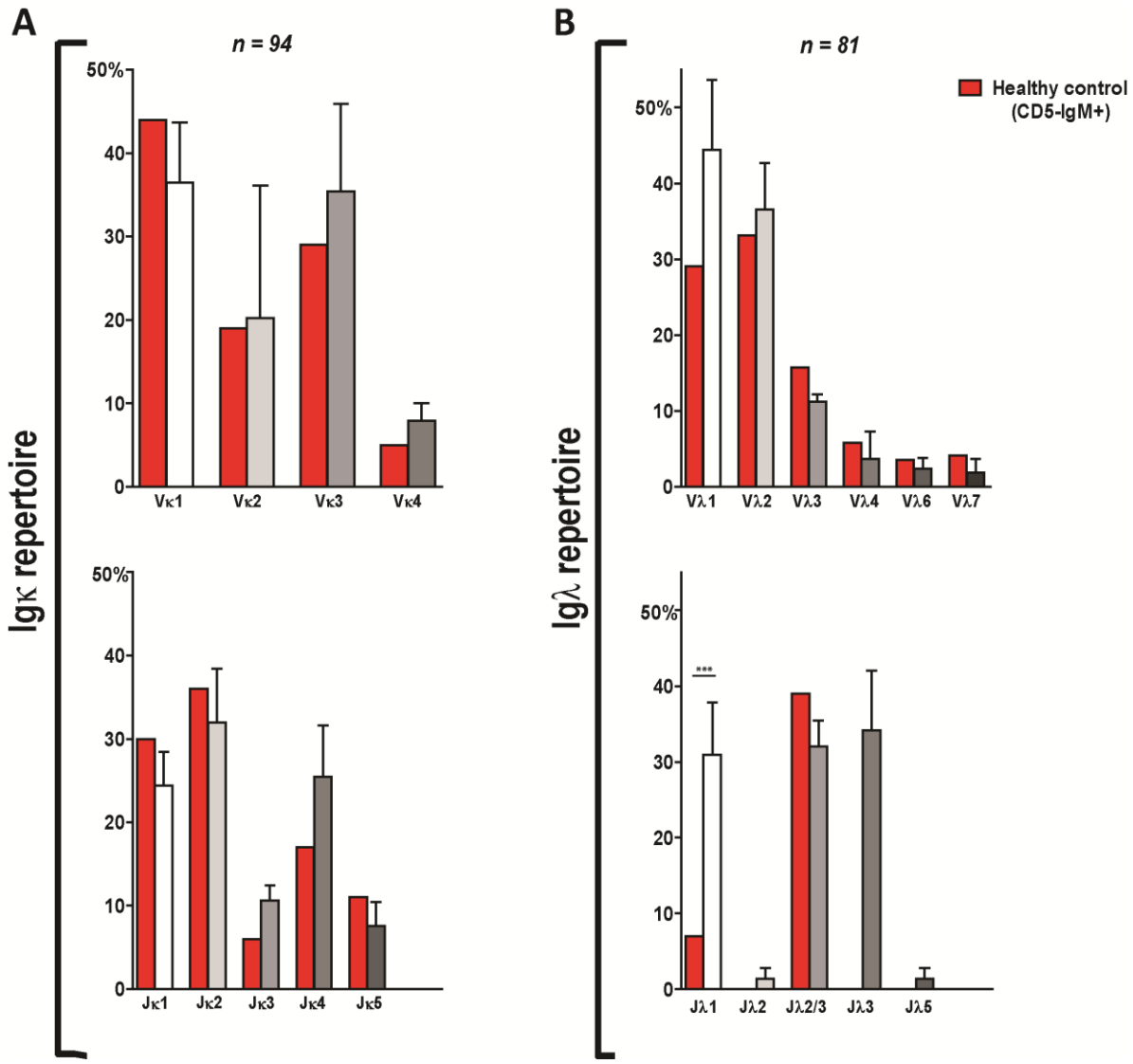

Figure 6.4 IgL gene analysis of single CD19+ synovial B cells from two RA patients.

Single CD19+ synovial B cell antibodies were analysed for IGK **(A)** and IGL **(B)** V family and J gene usage. The red bars indicate the VH and JH gene repertoire of peripheral blood naïve B cells [172]. The absolute number of sequences analysed is reported over each graph. Each bar in the graph indicate the mean + standard error of mean (SEM) for individual patients. *P* values compare data from RA synovial B cells to peripheral blood naïve B cells and it was calculated using the chi-square test (*** $P < 0.001$).

6.5 Somatic diversity and clonal relationship of synovial B cells.

According to the isotype, the synovial B cell clones showed an increasingly high number of somatic mutations in their V region (IgM < IgG < IgA). V genes of IgM+ synovial B cells carried on average 4 IGH gene mutations, IgG+ and IgA+ synovial B cells carried on average 15 and 17 IGH gene mutations, respectively (**Figure 6.5A**). Synovial B cells carried on average 18 IGK and 8 IGL V gene mutations (**Figure 6.5B**). All CD19+ B cell antibodies showed high ratios of replacement to silent mutations in CDR1 and CDR2 compared with those in FR1-3 regions (**Figure 6.5C**). This difference was less marked in the IgM+ antibodies.

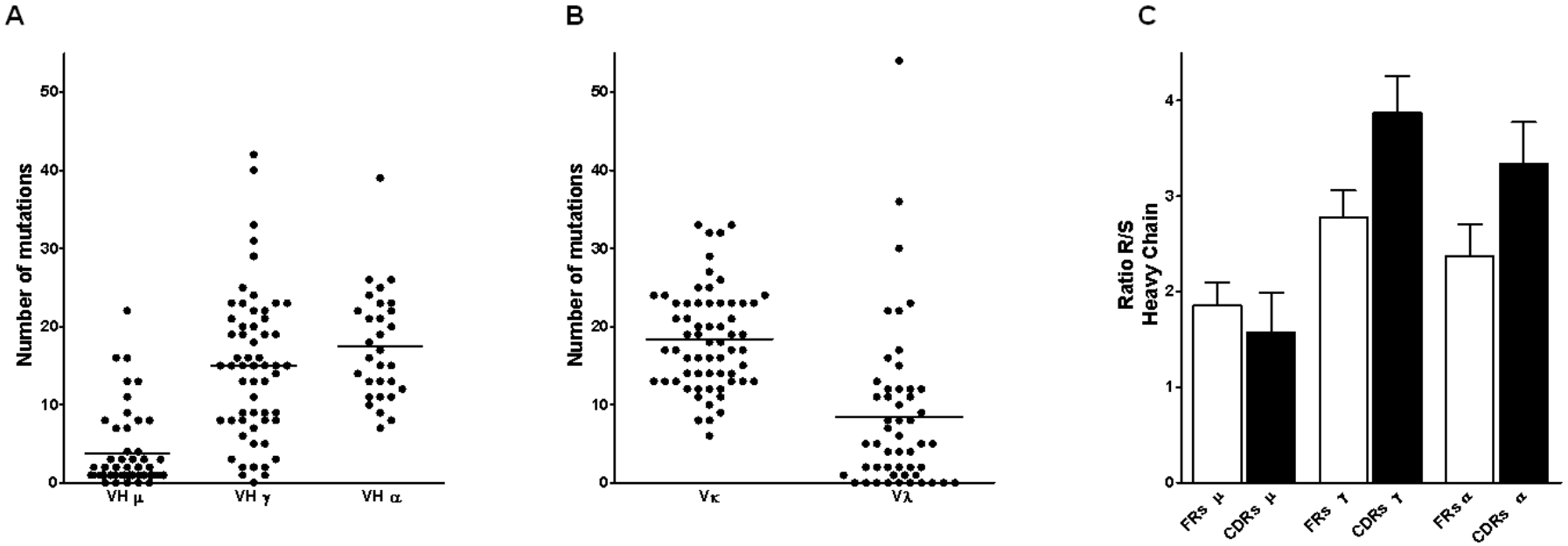

Figure 6.5 Number of somatic mutations and analysis of replacement (R) and silent (S) mutations ratio in FR and CDR regions of CD19+ synovial B cells.

The Ig gene sequences of CD19+ synovial B cells were analyzed for absolute numbers of somatic mutations in VH genes for IgM, IgG and IgA individually **(A)**, VL genes (κ and λ) **(B)**, and frequency of replacement and silent mutation ratio in FR (white) and CDR (black) regions for IgM, IgG and IgA individually **(C)**.

Ig sequences of the heavy chain are determined by a specific V(D)J rearrangement which defines a unique CDR3. Further diversity is introduced by N nucleotide addition during gene rearrangement and by point mutations through the process of SHM during clonal expansion of the B cells, as described in Chapter 1.1. The latter event increases the antigen binding affinity and brings to the formation of clonally related B cells. Thus, the Ig gene of these clonally related B cells derives from a single germline rearrangement and is characterised by the same V, D and J gene usage. The analysis of the VH gene repertoire of RA synovial B cells showed the presence of clonally related Ig genes. In particular, two sets of synovial B cells expressed the same V(D)J rearrangement with the same CDR3 and a different pattern of somatic mutations in the sample RA1. In this patient clones IgA11 with IgA82 and clones IgA88 with IgA91 were highly diversified from germline sequences but differed by one single mutation (**Figure 6.6A**). This suggests that in the joints of this patient IgA+ B cells underwent intra-synovial diversification. Similarly, two clonally related B cells were found in the sample RA2 (IgG35 and IgG80) and three in RA3 (IgG47, IgG80 and IgG91) (**Figure 6.6A**). Moreover, in sample RA2 two sets of proliferating clones (IgG33, IgG48 and IgG88 as well as IgG5, IgG35, IgG39, IgG83 and IgG91) were characterised by the same V(D)J rearrangement, identical CDR3 and the same pattern of somatic mutations. These data support the notion that the RA synovium is characterised by an active proliferation of synovial B cells due to an antigen-selected differentiation within the synovial tissue, as originally suggested by the Berek's group [163].

Interestingly, we found shared heavy CDR3 (HCDR3) regions between the two RA patients (**Figure 6.6B**). Two clones from RA1 (IgM12 and IgA64) shared two HCDR3

regions with RA2. IgM12 shared the same CDR3 with IgG33, IgG48 and IgG88 from RA2 (VH3-15, D 2-21, JH 5; CDR3=HFESCGGDCSNW). IgA64 shared the same CDR3 with IgG5, IgG35, IgG39, IgG83 and IgG91 (VH 3-9, D 3-22, JH 4; CDR3=DISSYDDTSGYYYN). Three clones from RA3 (IgG47, IgG80, IgG91) shared one HCDR3 with two clones from RA2 (IgG35, IgG80) (VH 3-48, D 3-22, JH 4; CDR3=VHMYYYDSSGYYYDDY). The presence of B cell clones with identical CDR3 but different V(D)J among different RA patients strongly suggest that common antigens may be driving humoral (auto)immunity within the joints and provide a fascinating scenario whereby identification of such antigenic forces may provide novel critical clues regarding RA pathogenesis.

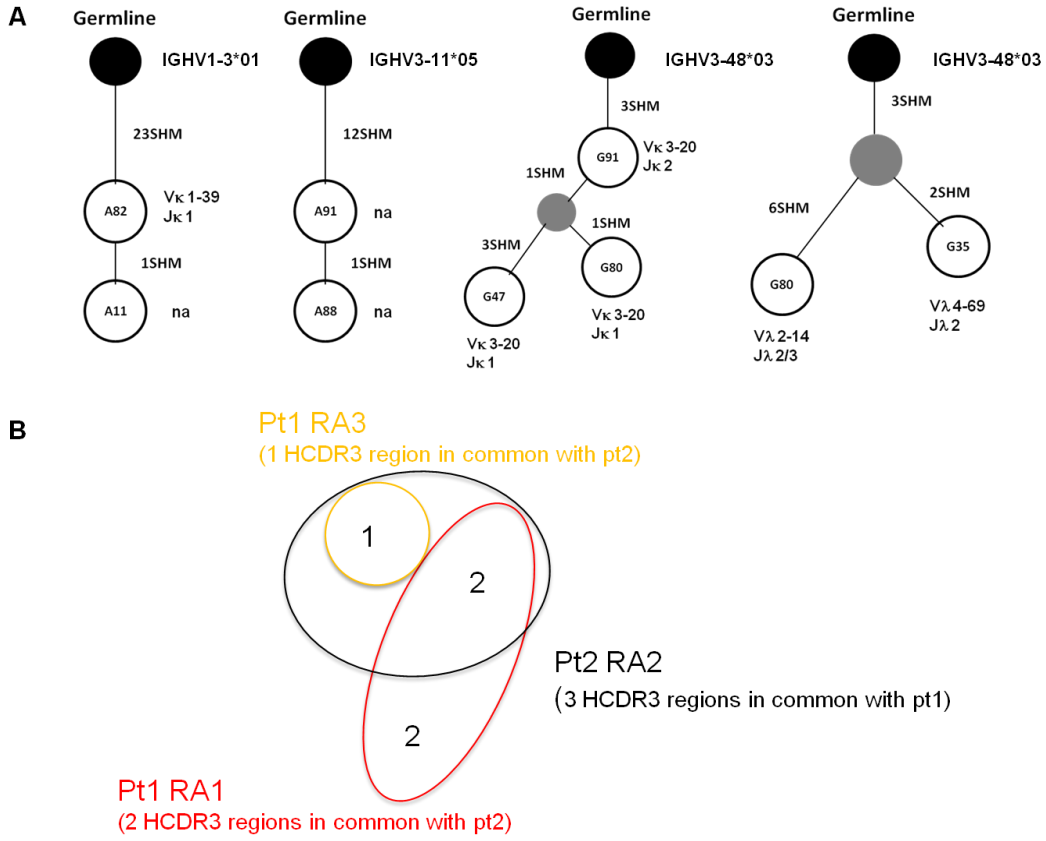

Figure 6.6 Lineage tree analysis of synovial B cells.

Schematic presentation of the sequence analysis of single CD19+ B cells in RA synovial tissue sample from two patients is shown. **(A)** Genealogic trees generated by comparison of Ig VH sequences of synovial B cells from sample RA1 and sample RA2. The synovial B cell clones are depicted as white circle, the putative common progenitor as grey circle and the germline sequences as black circle. The number inside the circle corresponds to the name of the clone and the number beside the line represents the additional mutation acquired. At the bottom of each clone the correspondent Ig VL sequence are indicated. "na" means not applicable and indicates those genes that we could not amplify. **(B)** Venn diagram represents the number of shared clones displaying an identical heavy chain CDR3 region between the different patients and samples.

6.6 Recombinant synovial B cell antibodies reactivity profile towards citrullinated antigens.

Out of all single CD19+ B cells isolated from the RA synovium, I manage to clone the matching IGH, IGK, and IGL chains genes of 66 individual B cells into specific expression vectors and produced the recombinant monoclonal antibodies *in vitro* (for full details of the repertoire of the synovial B cell antibodies see **Table 6.1**).

These antibodies have been used to investigate whether the RA synovial B cell antibodies were reactive against citrullinated antigens using a specific diagnostic ELISA test which is based on the detection of autoantibodies in human serum or plasma towards a synthetic cyclic peptide containing modified arginine residues (CCP2 peptide). This test has a sensitivity of 78% and a specificity of 99%, as reported by the manufacturer. Of the 66 antibodies, 59 were tested in the anti-CCP ELISA as 7 antibodies could not be used since their concentration was too low for the test (<1 μg/ml).

As shown in **Figure 6.7**, I observed a significant reactivity toward cyclic citrullinated peptides in 3 out of 59 RA synovial B cell antibodies (2 IgA and 1 IgG) and low reactivity in 5 more clones (3 IgG and 2 IgM). Of relevance, one of the 3 highest RA monoclonal antibodies positive for CCP (RA2 IgG80) was one of the clones clonally related with the RA2 clone IgG35 (**Figure 6.6A**) and it shared the same CDR3 with three clones from RA3 (**Figure 6.6B**).

These results, if confirmed in more refined studies (i.e. by testing the antibodies on specific citrullinated proteins (or derived peptides) associated with RA such as

vimentin, fibrinogen, etc.) would identify specific CDR3 regions shared among different RA patients responsible for the binding of B cells to citrullinated proteins (similarly to the HLADRQ “share epitope”).

Table 6.1 IgH and L gene repertoire analysis of CD19+ synovial B cells from RA patients.

Clones highlighted in grey are those expressed as recombinant antibodies. (-) and (+), negative and positive charges within the heavy chain CDR3; length, number of amino acids within the CDR3.

RA015/11	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length
μ														
88	4-4	3-10	2	2	EVPTPYFDL	0	9	κ	1-39	2	0	QQSYSTPYT	0	9
94	1-3	4-17	3	4	GGEDGYGDSYNAFDL	0	15	κ	1-5	1	0	QQYNSYSWT	0	9
γ														
58	3-64	1-26	5	1	EIVGANRWVPVGP	1	13	κ	3-15	1	0	QQYNNWPQST	0	10
68	4-31	6-25	6	2	AISWADGYYMDV	0	12	λ	1-40	3	1	QSYDSSLSGSV	0	11
69	1-24	5-5	6	1	ASSPFYYFYSMDV	0	13	λ	2-23	3	0	CSSAGSSAVV	0	10
96	3-30	1-1	5	3	DPQNENWSPPYNWFDP	0	16	κ	1-39	2	0	QQSHSIPYT	1	9
α														
48	3-66	3-3	4	3	EGDLWSGSIDY	0	11	λ	1-44	1	2	AAWDDSLNGRV	1	11
63	4-34	3-3	5	2	APQRFVEWLFWFDP	1	14	κ	1-39	2	0	QQGYTALYN	0	9
74	4-59	5-24	6	1	GSAGGGYYYYYGMDV	0	15	λ	1-40	2/3	1	QSYDSSLSGSGV	0	12
81	1-2	4-17	3	1	GAYGDPLHI	1	9	λ	3-21	1	3	QVWDSSFDRPD	1	11
82	1-3	2-2	4	3	DRDIVVVPTARSLGYSYGSFDS	2	22	κ	1-39	1	0	QQSYTNPRT	1	9
83	4-4	2-21	3	2	GPGTATSEAFDI	0	12	λ	2-23	3	0	CSHVIGGIWV	1	10
91	3-21	3-10	4	1	WRAGVPSYFDY	1	11	κ	1-33	3	0	QQYANVFT	0	8
95	1-58	3-16	4	1	GGSYVDY	0	7	κ	4-1	2	0	QQYYSNPYT	0	9
RA015/11	**VH**	**D**	**JH**	**(-)**	**CDR3(aa)**	**(+)**	**Length**	**κ/λ**	**Vκ/Vλ**	**Jκ/Jλ**	**(-)**	**CDR3**	**(+)**	**Length**
α														
11	1-3	2-2	4	3	DRDIVVVPTARSLGYSYGSFDS	2	22							
17	3-23	3-3	4	4	SPTDFWDDYLYYFDS	0	15	κ	3-20	1	0	QQYGSSPWT	0	9
53	1-2	3-10	5	2	SEGFTHYFDP	1	10	κ	3-15	1	1	QQYNDWPQT	0	9
62	3-48	6-25	4	2	EGHNSGYDY	1	9	κ	3-11	3	0	QQRYHWPPFT	2	10
64	3-9	3-22	4	3	DISSYDDTSGYYYN	0	14	κ	3-11	1	0	QQRSNWPGT	1	9
66	4-4	6-6	3	3	KGTYSTDSYDGFDI	1	14	λ	2-23	1	0	CSYAGSSTLYV	0	11
72	1-69	3-10	5	2	ELLRGDVVPAF	1	11							
75	3-33	3-16	4	2	DVGITGMINTFDY	0	13							
80	3-9	2-8	5	2	DISRGGSSAVFEF	1	13							
86	3-23	5-24	4	2	GDGYNPGVFFDY	0	12							
88	3-11	2-2	5	1	QPWGSTNWFDP	0	11							
91	3-11	2-2	5	1	QPWGSTNWFDP	0	11							
95								λ	1-44	3	2	ASWDDSLNVWV	0	11
μ														
3	1-18	6-6	4	3	DDSYSSSFDY	0	10							
9	3-23	4-17	6	1	GQSHSWSAMDV	1	11	κ	1-17	4	0	LQHNSYPLT	1	9
12	3-15	2-21	5	2	HFESCGGDCSNW	1	12	λ	3-21	2/3	2	QVWDSSSDHPGV	1	12
19	1-2	5-5	6	3	VGGGRQLWLKDNYDYFYMDV	2	20	κ	3-20	2	0	QQYGSSHT	1	8
22	4-59	2-2	2	1	IPAAPSYWYFDL	0	12							
23	3-11	6-19	4	1	SGQQWPWDY	0	9							
70	3-23	6-19	4	1	LAFVAATWRGPFDS	1	14	κ	1-16	4	0	QQYYGYPPT	0	9
74								κ	3-15	2	0	QQYYRWPPYT	1	10
78	3-30	2-21	6	3	ELGFPYCGGDCFSSMDV	0	17							
83	3-48	6-13	4	2	DMPHFLYSSRWYPFDY	2	16	κ	1-9	4	0	QQLNSYPLT	0	9
87	5-51	5-5	4	3	AYGYIWENSRYPENFDY	1	17	κ	4-1	4	0	QQYYTTPLT	0	9
89	3-11	3-9	4	2	AYSDILTGSPYDY	0	13	κ	3-15	4	0	QQYNNWPPLT	0	10
90	4-4	2-15	4	1	LTSKLGYCSGGSCYPYFDY	1	19	λ	1-40	1	1	QSYDSSLSGSYV	0	12
92	1-2	3-22	3	3	AGYYYDSSGYLPDAFDI	0	17	λ	1-40	1	1	QSYDSSLSGSHYV	1	13

RA056/11	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length
α														
23	4-59	6-19	5	2	LIAVAGTSDWFDP	0	13	λ	1-44	3	2	AAWDDSLNGPV	0	11
41								κ	3-15	1	0	QQCNNWPLT	0	9
70								λ	3-25	1	1	QSADSSGTHV	1	10
95	3-23	3-9	5	1	VPHQLVPIWFDP	1	12	λ	2-14	1	0	NSISSTSTNNV	0	11
96	3-30-3	5-24	3	2	DARGVRNAFDL	2	11	κ	1-17	1	0	LQHNSFPWT	1	9
								λ	7-43	3	1	LLYYDGGLWV	0	10
γ														
29	1-8	3-3	4	1	AAGVGVALDY	0	10	κ	3-20	3	1	QHYESSPPVFT	1	11
33	3-15	2-21	5	2	HFESCGGDCSNW	1	12	λ	4-69	1	1	QTWDTGIQV	0	9
35	3-48	3-22	4	3	VHMYYYDSSGYYYDDY	1	16	λ	2-8	1	0	SSYAGSNNYV	0	10
45	3-30	2-2	3	2	PHRLLDSCSSTSCYVVAFDL	2	20	λ	4-69	1	1	QTWDTGIQV	0	9
56	3-23	6-19	4	1	GTLSGFATTFDY	0	12	λ	2-14	1	0	SSYTSSSSLLYV	0	12
60								λ	1-44	3	2	AAWDDSLNGPV	0	11
66	4-39	1-26	4	1	RHIGRHYYFDY	4	11	λ	2-11	3/2	0	CSYVGSYTVA	0	10
68	4-61	6-6	6	2	DASIAARPPWGMDV	1	14	λ	2-23	3	0	CSYAAGNTRV	1	10
72	3-23	4-4	4	0	GARPSPSNYLYY	1	12	λ	1-44	3	2	AAWDDSLNGPV	0	11
76	1-69	3-3	5	2	VRITIFGVVMVKSDNWFDP	2	19	κ	3-15	2	2	QQYNNLYT	3	9
78	4-4	6-13	3	3	KGTYSTDSYDGFDI	1	14							
80	3-48	3-22	4	3	VHLYYYDSSGYYYDDY	1	16	λ	2-14	3/2	0	SSYTSSSTVV	0	10
83	3-9	3-22	4	3	DISSYDDTSGYYYN	0	14	λ	1-51	3/2	1	GTWDSSLSAVV	0	11
87								κ	3-15	4	0	QQYNAWPLIT	0	10
								λ	1-44	3	2	AAWDDSLNGPV	0	11
91	3-9	3-22	4	3	DISSYDDTSGYYYN	0	14	κ/λ	3-20	4	0	QQYGSSPLT	0	9
μ														
54	5-51	3-22	4	2	RYYYDSSGYTTFDY	1	14							
89	3-15	3-3	4	2	LGGYYDFWSGYYRIDY	1	16							
90	3-33	3-3	6	3	EAPIYDFWSGYRPYYYYYMDV	1	21							

RA056/11	VH	D	JH	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length
α														
35														
58	1-3	2-2	4	0	SLYCSTHSCSFLHLY	2	15	κ	3-20	2	0	QQYGSSPGT	0	9
93	4-4	3-3	4	1	TFWSGSYSRYFDS	1	13	κ	3-15	5	0	QQYYNWPPIT	0	10
γ														
5	3-9	3-3	4	3	DISSYDDTSGYYYN	0	14							
6	4-31	3-10	4	2	AVNVLLRFGELRHYFDQ	3	17	κ	3-20	5	0	QQYGSLPLI	0	9
12	4-59	4-17	4	2	YGVDYFDY	0	8	λ	1-51	2/3	1	GTWDSSLSAVV	0	11
20	5-51	3-22	6	2	QGYYDRSPRPHYMDV	3	15	κ	3-11	1	0	QQRSNWPPT	1	9
21	3-30	2-2	4	1	ETCSPTNCYPRN	1	12	κ	3-11	4	0	QQRSNSLT	1	8
23	3-33	6-25	4	1	VTSRVVAAAGGYFDH	2	15	λ	3-9	2/3	1	QVWDISSVV	0	9
26	1-69	5-5	4	3	EGTAMVLEGFLDY	0	13	λ	1-44	3	1	AACDGSLNGHVV	1	12
27	4-4	2-15	6	1	GFVVVPSAMKQGNSRIPYYSYYMDL	2	25	λ	2-8	1	0	SSYAGSNNYV	0	10
35	3-9	3-22	4	3	DISSYDDTSGYYYN	0	14	κ	3-15	4	0	QQYSYWFT	0	8
								λ	1-51	2/3	1	GTWDSSLSAVV	0	11
36	4-39	1-26	4	1	RHIGRHYYFDY	4	11	λ	2-14	2/3	0	SSYTSSSTLV	0	10
39	3-9	3-22	4	3	DISSYDDTSGYYYN	0	14	λ	1-47	3	2	AAWDDSLSGWV	0	11
42								κ	4-1	4	0	QQYYSTLALT	0	10
45	3-30	2-2	6	2	DIVVVPAATSLLGGYYYYYMDV	0	22	κ	1-39	4	0	QQSSTTPLT	0	9
48	3-15	2-21	5	2	HFESCGGDCSNW	1	12	κ	1-39	2	0	QQSYSTPYT	0	9
54	1-46	1-7	6	3	DGLEARRTTSSHPHYYMDV	4	19	κ	3-11	4	0	QLRSNWRT	2	8
56	4-34	6-6	6	1	KKGRVGIAYMEV	3	12	κ	1-39	3	0	QQSFSMPFT	0	9
59	3-9	6-13	4	2	DSAAGTPVYFDY	0	12	κ	1-33	4	1	QQYDNLPLT	0	9
								λ	1-44	3	2	AAWDDSLNGPV	0	11
63	5-51	3-3	5	1	HRGPTTIFGVAIGAFDP	2	17	λ	1-44	3	2	AVWDDSLNGPV	0	11
66	-30-4(4-31	3-9	5	3	GVFDNFFNRRLETNWFDP	2	18	κ	3-11	3	0	QQRSSWPPT	1	9
72	3-15	2-21	5/4	2	HFESCGGDCSNW	1	12	λ	6-57	2/3	2	QSYDNDNLWV	0	10
75	4-59	5-5	2	2	TPYPPLDWYFDL	0	12	κ	4-1	1	0	QQYYITPPT	0	9
88	3-15	2-21	5/4	2	HFESCGGDCSNW	1	12	λ	1-44	1	2	AAWDDSLNGYV	0	11
94	3-30	3-10	4	3	EVREYTDY	1	8	κ	3-11	4	0	QLRSNWLLT	1	9
95	3-23	1-26	4/3	0	LVGITHLSAAPWT	1	13	λ	2-23	3	0	CSYAGTWV	0	8
μ														
2								λ	1-44	3	2	AAWDDSLNGPV	0	11
3	3-21	3-22	3	3	DYYDSSGYLSAFDI	0	14	λ	1-44	3	2	AAWDDSLNGWV	0	11
4	1-18	4-17	4	2	ASYGDYSDY	0	9	κ	1-13	2	0	QQYYSTPPYT	0	10
								λ	1-44	3	2	AAWDDSLNGPV	0	11
9	3-23	3-10	3	4	CETGERRWYYYGSGTIREAFDI	3	22	κ	3-11	4	0	QQRSNWPPT	1	9
15	1-69	4-17	4	1	DQATTVTTRVWFVY	1	14	λ	1-47	3	2	AAWDDSLSGRV	1	11
24	1-3	2-2	4	0	VGIVVVPAAALGY	0	13							
25								λ	1-44	3	2	AAWDDSLNGPV	0	11
34	3-21	6-13	5	3	PRQLGSVWFDP	4	12	κ	1-9	4	0	QQLNSYPLT	0	9
38	3-33	6-13	4	2	DRSSSWYFDH	2	10	λ	2-14	3	0	SSYTSSSTWV	0	10
39								λ	1-47	3	2	AAWDDSLSGWV	0	11
41	3-23	3-10	4	1	GSGTFDY	0	7	λ	3-21	1	2	QVWDSSSDHYV	1	11
48	4-31	6-6	6	1	VSLNSSSSLIHYYYYMDV	1	18	κ	1-39	2	0	QQSYSTPYT	0	9
67	3-30	5-12	6	3	DYTAWLLNEYYYYGMDV	0	17	λ	1-47	3	2	AAWDDSLSVWV	0	11
81	3-64	3-3	4	3	EYDFWSGYYYRGATRTTPNFDY	2	22	κ	3-15	2	0	QQYNNWPLWT	0	10
91	1-69	5-5	4	3	DLVDPHLLTHGFDY	2	14	κ	3-15	2	0	QQYNGWPLPMYT	0	12

RA057/11	VH	D	HJ	(-)	CDR3(aa)	(+)	Length	κ/λ	Vκ/Vλ	Jκ/Jλ	(-)	CDR3	(+)	Length
α														
6	3-30	2-15	4	0	AHIVVVVAASYYFAY	1	15							
12	3-11	3-3	4	1	SFWNGYHFDY	1	10	λ	2-14	2/3	0	SSYTTSGTYVV	0	11
25	3-7	6-13	6	3	DQVEQQLVLGYFYYYYMDV	0	19	λ	1-44	1	1	AAWDASLKV	1	9
43	3-33	4-17	6	2	ADYGNSYYYYMDV	0	13	λ	2-8	1	0	SSYAGSNNYV	0	10
47	3-7	3-3	4	4	DPRAYDYWSGYYEGYFDY	1	18	κ	3-20	1	0	QQYGSSPGT	0	9
								λ	2-23	3	0	CSSASFTISWV	0	11
γ														
3	4-39	2-15	5	1	LFGPGIPGWWLDP	0	13	κ	3-20	4	0	QQHGSSRGLT	2	10
5	1-2	5-5	5	1	TSMGYTSTWAYNWFDP	0	16	λ	2-23	2	2	CLYAGEEVL	0	9
10	4-34	5-5	6	2	GVRGGYTSDSFPLYYFMDL	1	19	κ	1-12	5	0	QQANSFPVT	0	9
26	3-21	1-14	3	1	TPNWNHRGGAFDI	2	13							
29	1-69	3-16	6	1	GRLPWRYMDV	2	10							
31	1-18	2-15	6	1	TPRYYYYYMDV	1	11							
36	4-28	2-2	6	1	RALYHHYMDV	3	10	κ	3-20	2	0	QQHGSSPYT	1	9
								λ	2-8	2/3	0	SSYAGVHTVI	1	10
47	3-48	3-22	4	3	VHLYYYDSSGYYYDDY	1	16	κ/λ	3-20	1	0	QQYGSSPGT	0	9
50	3-9	3-16	6	1	GSYRYYYYCIDV	1	12	κ	3-20	2	0	QQYGSSPVYS	0	10
52	4-31	3-3	3	2	LVGIFGGDAFDI	0	12	λ	1-51	2/3	2	EVWDSGLSVRL	1	11
57	3-48	6-19	4	1	AGLSGSGPFDY	0	11	λ	1-47	2/3	2	AAWDDSLSGVV	0	11
68	3-30	6-19	4	1	PAVVGASLHFDY	1	12	κ/λ	3-20	2	0	QQHGSSPYT	1	9
69								κ	1-27	1	0	QKYNSAPRT	2	9
72	3-23	1-26	5	1	HWDS	1	4	λ	1-44	1	1	SAWDNSLNGYF	0	11
77	3-72	1-7	6	2	VKATYGWKINVDQFLDV	2	17	κ/λ	1-39	1	0	QQSFRTPWT	1	9
78	3-21	3-3	4	1	LGYDFWSGHRH	3	11	λ	6-57	1	2	WSYDNYQEI	0	9
80	3-48	3-22	4	3	VHLYYYDSSGYYYDDY	1	16	κ	3-20	1	0	QQYGTSPWT	0	9
								λ	1-47	2/3	2	AAWDDSLSGVV	0	11
89	4-59	??	2	2	TPYPPLDWYFDL	0	12	κ/λ	1-47	2/3	2	AAWDDSLSGVV	0	11
91	3-48	3-22	4	3	VHLYYYDSSGYYYDDY	1	16	κ	3-20	2	0	QQHGSSPYT	1	9
								λ	2-14	2/3	0	SSYTSSSTRRVV	2	12
93	3-74	3-3	6	5	DGGEAYDFWSDNHRFYFYYYMDV	2	23	λ	2-14	2/3	1	SSYTTSSDLV	0	10
μ														
2	1-46	4-23	4	3	FGRHDYGGKDDY	3	12	κ	1-33	2	1	QQYDNLPYT	0	9
17	4-31	3-10	6	3	DQITMVRGGDGQNYYYYYMDV	1	21	κ/λ	1-39	3	0	QQSYSTPPLST	0	11
20	3-9	6-19	4	2	DSRRKSIAVAGAYFDY	3	16	κ/λ	2-8	1	0	SSYAGSNNYV	0	10
21	3-23	3-9	6	3	DGRPSTIFWDYMDV	1	14	λ	2-14	2	0	*RSYTTIINNLST*	1	11
28	3-21	2-21	4	3	DVGDIVVVTASLDY	0	14	λ	1-44	2	2	AAWDDSLNGVV	0	11
29	3-23	5-5	3	1	GIQLWPGGAFDI	0	12							
30	3-30	3-10	4	2	DLAPGFTSAFDY	0	12	κ	4-1	4	0	QQYYTLPLT	0	9
32	3-23	3-22	4	3	DGYYDRRGGTVDY	2	13	κ	3-15	1	0	QQYNNWPPWT	0	10
								λ	2-8	1	0	SSYAGSNNYV	0	10
35	4-34	6-13	4	1	GWAYSSSWYRRMISFDY	2	17	κ/λ	3-20	2	0	QQHGSSPYT	1	9
44	1-46	3-22	4	2	VGGGYYDSSGGALDY	0	15	κ	1-5	1	0	QQYNSYPWT	0	9
48	3-23	5-12	4	2	VGYSGYDLRVYYFDY	1	15	λ	2-8	1	0	SSYAGSNNYV	0	10
51	4-59	2-21	3	2	RVGSPYCGGDCYPAFDI	1	17	κ	1-8	3	0	QQYYSYPT	0	8
56	5-51	2-2	6	2	ILVDCSSTSCYYYYYMDV	0	19	λ	3-25	2	1	QSADSSGLV	0	9
58	3-30	1-7	4	1	QWGATGTLDY	0	10	κ	4-1	3	0	QQYYSTPT	0	8
61	3-21	6-13	4	1	GGSSWYYFDY	0	10	λ	1-44	3	2	AAWDDSLNGWV	0	11
62	3-7	3-3	4	1	ELFHILSY	1	8	κ	1-33	2	1	QQYDNLPLT	0	9
								λ	1-44	2/3	2	AAWDDSLNGPV	0	11
63	3-11	6-13	6	2	EMGGSSWSIYYYYYYMDV	0	18	κ	1-5	2	0	QQYNSSPYT	0	9
								λ	1-47	2/3	2	AAWDDSLSGVV	0	11
67	4-59	7-27	3	2	RESSRLGNAFDI	2	12	λ	2-23	2/3	0	CSYAGSSTL	0	9
71	1-18	5-24	4	2	DLNSYYFDY	0	9	κ	4-1	4	0	QQYYSTPLT	0	9
								λ	2-8	1	0	SSYAGSNNYV	0	10
75	3-21	1-7	2	1	AGNWNYYWYFDL	0	12	λ	2-8	1	0	SSYAGSNNYV	0	10
82	1-24	1-26	3	1	PIVLGAFDI	0	9	κ	3-20	2	0	QQYGSSPPYT	0	10
								λ	2-23	2	0	CSYAGSPV	0	8
89	1-18	2-2	6	1	RYCSSTSCYKGSYYYYYYYMDV	2	22	κ	3-20	4	0	QQYGSSPLT	0	9
								λ	1-47	2/3	2	AAWDDSLSGVV	0	11

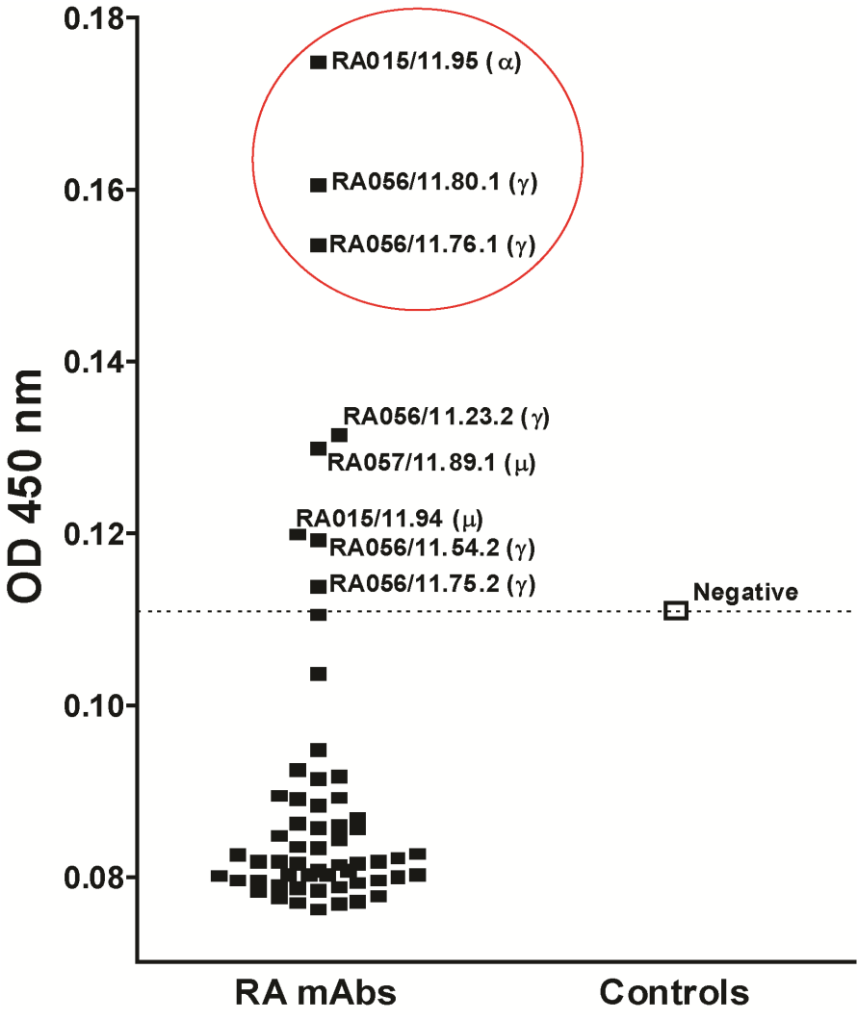

Figure 6.7 RA synovial B cell antibodies reactivity against cyclic citrullinated peptide.

Synovial B cell antibodies (n=59) from two RA patients were tested for reactivity against cyclic citrullinated peptide by ELISA. The graph illustrates the reactivity at a concentration of 10 μg/ml. The horizontal line shows the OD value for the negative control (human plasma negative for CCP) supplied by the ELISA kit.

Chapter 7 | Discussion

7 | Discussion

Sjögren's syndrome (SS) and rheumatoid arthritis (RA) are two common inflammatory autoimmune diseases characterised by peripheral B cell disturbances in the naïve and memory B cell compartments. The presence of profound B cell disturbances is a pivotal feature of patients with SS and RA and has been demonstrated to play a fundamental role in disease pathogenesis and clinical evolution [47, 186]. Both SS and RA are characterised by high affinity circulating autoantibodies and by the formation of functional ectopic lymphoid structures (ELS) in the respective target organs, i.e. the salivary glands and the joint synovium, respectively. In these structures autoreactive B cells can undergo antigen selection and affinity maturation. Furthermore, evidence that treatment with B cell depleting biological therapies (i.e., rituximab) is associated with clinical improvement in RA patients (with extremely promising results also in pilot clinical trials in SS) confirms the importance of autoreactive B cell activation in disease pathogenesis [2, 187]. In SS the exact stage at which errors in tolerance checkpoints accumulate is unknown, whereas it has been shown that RA patients are characterised by presence of defective central and peripheral tolerance checkpoints [43].

Thus, in the first part of my work I aimed to investigate whether peripheral naïve IgD+ B cells with germline IgV H and L genes from patients with SS displayed an accumulation of autoreactive B cell receptors as one of the possible mechanisms leading to breach of B cell self-tolerance and development of enhanced B cell autoreactivity in SS.

In RA, as well as in SS, the nature of the antigens driving the humoral autoimmune response within the joint synovium and salivary glands characterised by ELS is still unknown. Their identification would provide novel essential information on the aetiology and pathogenesis of these autoimmune diseases. Therefore, in the second part of my work I aimed to identify the antigenic specificity of synovial B cells single-sorted from the synovial tissue of patients with RA with ELS.

7.1 Accumulation of circulating autoreactive naïve B cells reveal defects of early B cell tolerance checkpoints in patients with Sjögren's syndrome.

As mentioned, SS patients display presence of circulating immune complexes, hypergammaglobulinemia, serum organ-specific and non-specific autoantibodies, characteristic disturbances of peripheral B cell subsets, formation of ectopic lymphoid structures in the salivary glands, and increased risk of developing extranodal B cell lymphomas [47]. However, the mechanisms leading to breach of B cell self-tolerance and development of enhanced B cell autoreactivity in SS patients are still unclear. In order to address this aspect, as reported in Chapter 5, I employed a novel approach [33, 34], which allowed me to generate a large number of complete (i.e., IgH + IgL chains) recombinant monoclonal antibodies from single peripheral CD27-IgD+ B naïve cells bearing identical specificity to the original B cells. By these means I provided evidence for the first time that patients with SS display a significant accumulation of autoreactive naïve B cells in the peripheral blood compared to age and sex matched healthy individuals. Although the reactivity observed appeared to be generally of lower

affinity compared to antibodies previously generated from mutated switched memory B cells of SLE patients, evidence that unmutated germ line clones from SS patients display increased reactivity to Hep-2 cells (nuclear and/or cytoplasmic), ENA and dsDNA compared to HD clearly demonstrates a specific accumulation of self-reactive naïve B cells in SS patients.

In physiological conditions, a large proportion of self-reactive and polyreactive B cells which are normally generated in the bone marrow during B cell development (75% and 55%, respectively) are efficiently silenced at two major tolerance checkpoints before entering the peripheral compartment as naïve B cells [182, 188]. The first checkpoint removes most polyreactive B cells in the bone marrow (central tolerance checkpoint) [42, 189]. The second checkpoint in the periphery ensures that only a relatively small percentage of self-reactive and polyreactive mature naïve B cells (20% and 6%, respectively, as also confirmed in healthy donors in my study) survive [42, 189]. This physiological fraction of polyreactive and autoreactive naïve B cells is believed to form a reservoir of “natural” antibodies exerting a first-line protective role by recognizing invading pathogens [189, 190].

Thus, the evidence from my work that an increased proportion of autoreactive CD27-IgD+ naïve B cells accumulate in the peripheral blood of SS patients strongly supports the conclusion that early B cell tolerance checkpoints are significantly impaired in SS. It remains to be clarified whether this represents a primary event early in the pathogenesis of SS or is secondary to chronic inflammatory stimuli and whether the accumulation of autoreactive naïve B cells is a critical step towards the development of autoimmunity in patients with SS. Although this is the first evidence of such defects in

SS, previous reports demonstrated similar abnormalities in patients with SLE, RA and type 1 diabetes, suggesting that defects in central/peripheral B cell tolerance checkpoints are a common denominator of autoimmune diseases [177, 182]. Although the exact mechanisms regulating the early selection of auto/polyreactive B cells are not fully characterised in humans, it has been suggested that AID plays a fundamental regulatory role in early B cell tolerance as AID-deficient patients display similar accumulation of autoreactive naïve B cells [191] and AID-deficient mice develop autoimmunity [192].

An important observation from this study, which confirms previous evidence in SS and other autoimmune diseases [35, 41, 42, 189] is that the analysis of IGH, IGK and IGL chain gene repertoire showed no significance differences in gene family usage between naïve B cells from SS patients and HD, suggesting that family usage *per se* is unlikely to be responsible for the observed accumulation of autoreactive B cells in the periphery. However, I did notice a significantly higher frequency of positively charged amino acids within the IgH CDR3s, a feature commonly associated with autoreactive antibodies [33]. The increase of positively charged CDR3s in autoreactive naïve B cells with germ line sequences is of interest as accumulation of positively charged amino acids normally results from clonal diversification in germinal centres in SLO.

In keeping with the evidence that naïve B cells with germ line V genes can display autoreactivity in SS, previous reports demonstrated that one of two anti-Ro52 monoclonal antibodies and 4 out of 10 RF monoclonal antibodies generated from SS patients via EBV transformation and hybridoma technology were characterised by

completely unmutated germ line genes [193, 194]. Among the antibodies that I generated from naïve B cells of SS patients, 6 clones displayed a low reactivity towards Ro/SSA and/or La/SSB although at levels significantly higher compared to HD, a result that was confirmed in two independent assays (ENA screening and anti-Ro/La ELISA). Although circulating autoantibodies against Ro/SSA and La/SSB antigens are not known to be cross-reactive, some of the SS recombinant monoclonal antibodies showed a cross-reactivity among these antigens (**Figure 4.6**). Since the reactivity was confirmed in two ELISA assays independently, the cross-reactivity may be explained by the fact that these recombinant monoclonal antibodies could behave as polyreactive antibodies which are able to recognize structurally different antigens.

Thus, overall, the results provided during my PhD work together with previous data support the conclusion that a sizeable proportion of autoreactive B cells in SS patients resides within the naïve B cell population.

Although in this work I specifically focussed on CD27-IgD+ naïve B cells, there is strong evidence that patients with SS also display significant abnormalities in the peripheral CD27+ memory B cell compartment. Specifically, as also confirmed in this work and shown in Chapter 5, SS patients are characterised by a dramatic reduction in the percentage and total number of circulating CD27+ memory B cells, possibly as a result of accumulation in the affected salivary glands [82]. Within the inflamed salivary glands of SS, lesional memory B cells are characterised by high mutational load and evidence of ongoing clonal diversification, strongly suggesting a local antigen-driven process [72]. This is particularly evident in the context of ELS, which develop in 30-40% of SS patients [73, 141] and are characterised by the formation of functional germinal

centres promoting the selection and differentiation of autoreactive B cells, leading to generation of Ro/La immunoreactive plasma cells [73, 150, 195]. Importantly, my group and others previously showed that a subset of IgD+ B cells preferentially accumulate within ectopic B cell follicles in SS salivary glands [166]; furthermore, Le Pottier et al also showed that IgD+ B cells with a type-2 transitional-like phenotype infiltrating SS salivary glands are frequently autoreactive [166]. Thus, the demonstration that SS patients have an enlarged pool of autoreactive naïve B cells in the circulation may be relevant to SS pathogenesis as these cells can migrate from the periphery into the inflamed glands and give rise to B cell follicles where high affinity autoreactive clones can be selected and undergo differentiation in ectopic germinal centres (**Figure 7.1**).

In this regard, it is well known that in order to prevent selection of high affinity autoreactive clones a third tolerance checkpoint (the so called pre-germinal center checkpoint [189]) excludes self-reactive naïve B cells from entering germinal centre reactions, thereby avoiding their expansion and differentiation into long-lived plasma and memory B cells [189]. In physiological conditions in secondary lymphoid organs, autoantigen-binding B cells are excluded from entering B cell follicles because they down-regulate CXCR5 and are thus less responsive to CXCL13 compared to non-autoreactive B cells which compete for the same follicular niches [167]. As a result, autoreactive B cells accumulate at the boundaries between the T and B cell zone where they become anergic and/or die by apoptosis. However, this mechanism of follicular exclusion has been shown to be impaired in SS salivary glands, allowing autoreactive naïve and memory B cells to enter the ectopic germinal centres and

undergo clonal selection and affinity maturation [166], similarly to what has been described in SLO of patients with SLE [177]. Although the signals responsible for the regulation of CXCR5 expression on autoreactive B cells are unknown, it is possible to speculate that within ELS developing during chronic inflammation the generation of the suppressive, counter-immunoregulatory pathways normally active in SLO is impaired.

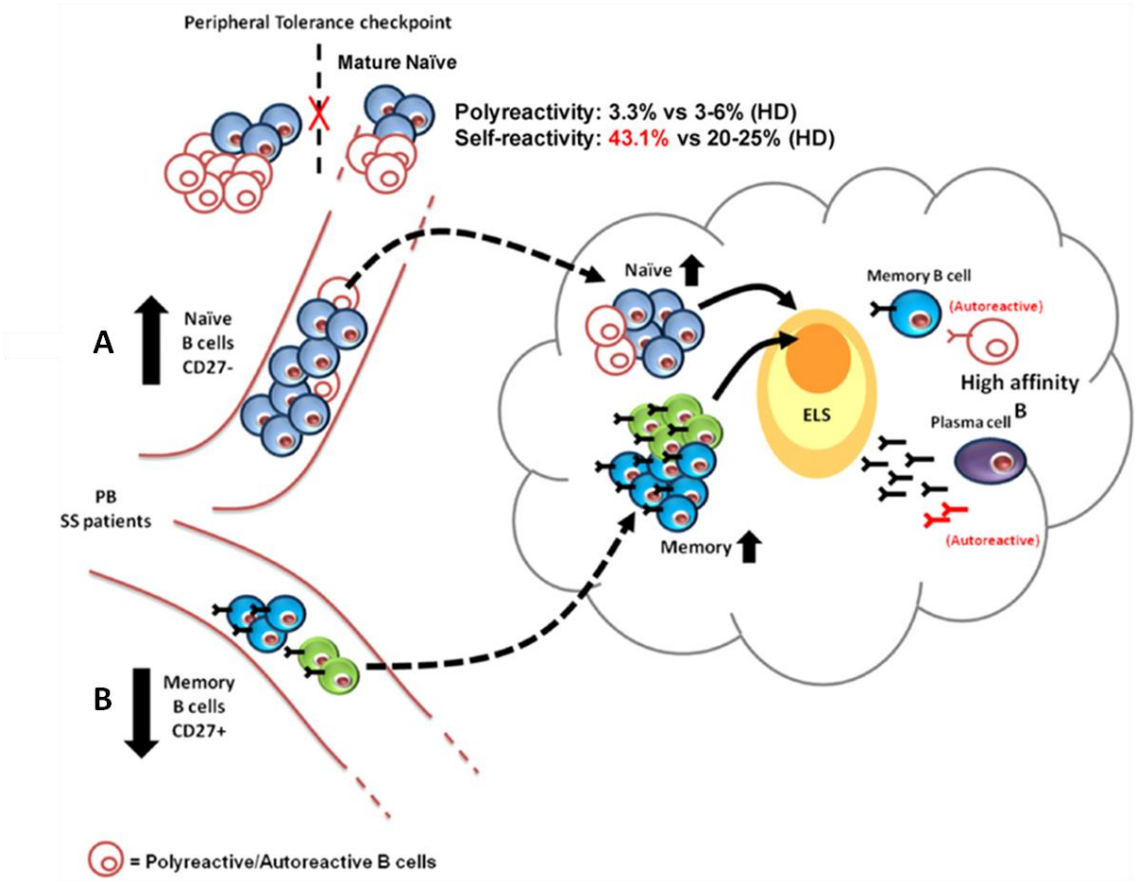

Figure 7.1 Peripheral self-tolerance checkpoint impairment in SS patients.

The cartoon shows how a defect in peripheral tolerance checkpoints could bring to the accumulation of autoreactive naïve B cells which can migrate from the periphery into the inflamed glands and possibly give rise to B cell follicles where high affinity autoreactive B cell clones can be selected and undergo differentiation in ectopic germinal centres leading to the production of autoantibodies producing plasma cells **(A)**. The reduction of circulating memory B cells in SS patients is explained as a result of their migration/accumulation in the affected salivary gland where lesional memory B cells can be selected and undergo further clonal diversification, thus contributing to the production of autoantibodies producing plasma cells **(B)**.

In summary, the work dedicated to the analysis of peripheral IgD+CD27- B cells in patients with SS demonstrates for the first time the accumulation of a larger proportion of self-reactive naïve B cells in the peripheral compartment strongly suggesting the presence of defective early tolerance checkpoints in patients with SS. It is possible to speculate that the continuous supply of newly generated autoreactive B cells could potentially contribute to SS pathogenesis by promoting and maintaining the autoimmune process either in SLO and/or within ELS. Furthermore, this work has potential implications in the understanding of the mechanisms responsible for the repopulation of autoreactive B cell clones following B cell depleting therapies in SS.

As reported in Chapter 4 and Chapter 5, SS patients are characterised by a distinctive distribution of peripheral B cell subpopulations. Analysis by several groups [47] confirmed by data from my own work, showed that pSS patients are characterised by normal total B cell counts in the peripheral pool but characteristic qualitative B cell disturbances with a predominance of CD27- naïve B cells and diminished peripheral CD27+ memory B cells [186], especially the circulating IgD+CD27+ memory unswitched subpopulation (**Figure 4.1**). These findings are in contrast with the normal pattern of peripheral B cells normally found in healthy individuals where CD27- naïve B cells and CD27+ memory B cells represent around 60-70% and 30-40%, respectively, of all CD19+ B cells [47]. To date, this altered distribution of circulating B cells seems to be unique for SS patients since patients with SLE have shown to have more circulating CD27+ memory B cells and reduced numbers of CD27- naïve B cells, whereas RA patients have shown a similar distribution of both populations in the periphery with a markedly increase of IgD+CD27+ memory unswitched B cells [47].

The reduction in circulating CD27+ memory B cells has been explained as result of migration to, or retention in, sites of inflammation since we and other groups observed an accumulation/retention of CD27+ memory B cells in the inflamed salivary glands of SS patients [82]. Molecular studies on B cells obtained from both peripheral blood and salivary glands of patients with SS have shown a polyclonal accumulation of memory B cells in the inflamed tissue characterised by a similar VH gene repertoire when compared to the peripheral counterpart. Moreover, lesional B cells have been shown to be characterised by heavily mutated Ig V region and shorter CDR3, supporting accumulation of memory B cells in the inflamed glandular tissue [47].

In parallel with the naïve B cells reactivity characterization, I also started to clone and express monoclonal antibodies from memory IgD-CD27+ switched and IgD+CD27+ unswitched B cells. As shown in Chapter 5, I analysed the Ig V gene usage of circulating memory switched and memory unswitched B cells isolated from peripheral blood of patients with pSS. This analysis provided no indication of clear abnormalities in Ig VH, Vκ and Vλ gene usage in SS patients, confirming data previously published [47]. Although results are not conclusive due to the relatively low number of memory B cells that can be cloned from the periphery of SS patients. I could not observe an altered level of polyreactivity in the circulating switched memory B cell antibodies comparing our data with previous analysis on memory B cells antibodies from normal individuals [40]. Conversely, the frequency of polyreactive memory unswitched B cell antibodies seemed to be increased compared to normal individuals. These results are extremely interesting as circulating IgM memory B cells bearing a marginal zone-like phenotype

[185] have long been implicated in promoting autoimmunity, chronic inflammation and evolve towards MALT lymphomas in SS patients [82].

In normal conditions, self-reactive memory B cells have been shown to be anergic or produce only low affinity non-pathogenic autoantibodies [40]. However, in susceptible individuals peripheral autoreactive memory B cells may eventually develop into autoantibodies producing plasma cells, either in SLO, followed by migration into the site of inflammation, or directly within ELS, and thus directly contribute to the development of autoimmunity [40].

Whether autoantibodies in SS play a pathogenic role is still unclear. Several autoantibodies against non-organ and organ specific autoantigens have been described in patients with SS. Among all of them, organ specific autoantibodies directed against the M3 muscarinic acetylcholine receptor (M3R) have been implicated in inducing impairment of salivary glands function [47, 87]. In particular, Tsuboi and colleagues showed that anti-M3R antibodies against the second extracellular loop of the M3R could be implicated in reducing salivary gland secretion by suppressing the increase in intracellular Ca^{2+} concentration involved in inducing salivary secretion [87]. Since the strategy used in this work allowed me to express recombinant monoclonal antibodies from memory B cells of SS patients, it will be interesting to determine whether the SS monoclonal antibodies produced are reactive towards the M3R. Several methods have been used to analyze anti-M3R antibodies. For instance, ELISA and Ca^{2+}-influx assays for the 2^{nd} extracellular loop of anti-M3R can be used, as previously reported [87]. Second, to clarify the pathogenic role of anti-M3R autoantibodies in SS, the monoclonal antibodies reactive against the M3R could be

transfer to immunodeficent mice in order to investigate the secretory function and pathology in the salivary glands [87]. These are part of the next steps of my investigation as detailed in the Future Plan section (Chapter 8).

7.2 Characterisation of recombinant monoclonal antibodies from single B cell isolated from the rheumatoid arthritis synovium.

In parallel with the characterisation of recombinant antibodies generated from naïve and memory B cells isolated for peripheral blood of SS patients, I also successfully generated large number of recombinant antibodies derived from single B cells isolated from synovial tissue of RA patients.

My group has recently provided the first direct evidence of ELS functionality in the RA synovium by demonstrating that these structures support the expression of activation-induced cytidine deaminase (AID) [111], the enzyme responsible for somatic hypermutation (SHM) and class switch recombination (CSR) of the Ig genes which normally take place in the germinal centres of SLO. In keeping with AID functionality, ELS also support the expression of Iγ-Cμ circular transcripts, which are specific transcribed by-products of CSR from IgM to IgG. Although synovial AID expression requires the formation of B cell follicles, also fibroblast-like synoviocytes can directly induce AID expression and circular transcripts in naïve B cells via release of BAFF and APRIL, two pivotal B cell survival and proliferating factors [196, 197]. Thus, stromal-immune cell interactions within ELS regulate B cell activation in the RA synovium and local plasma cell differentiation. This is indicated by the close correlation between ELS and synovial plasma cell number [160, 162] and by the analysis of Ig V gene repertoire

demonstrating clonal relationship between activated B cells, isolated using laser capture microdissection from synovial ELS, and surrounding plasma cells [163]. Furthermore, a significant proportion of CD138+ plasma cells migrating out of synovial ELS produce antibodies directed against the citrullinated but not the unmodified form of fibrinogen [111]. Accordingly, RA synovial tissues with AID+ ELS display sustained production of human IgG ACPA when engrafted into SCID mice in the absence of re-circulating cells, suggesting that synovial ELS can form self-sustained niches of autoreactive B cells which can differentiate into plasmablast/plasmacells and produce disease-specific autoantibodies within the target organ.

Additionally, these findings, which support a pathogenic role for ELS in RA, may also explain why drugs that effectively clear B cells from the bloodstream, such as the anti-CD20-specific monoclonal antibody Rituximab (RTX), do not always produce a marked clinical improvement in RA and might influence relapse/resistance to RTX therapy. However, despite peripheral B cell depletion is achieved in all cases and the treatment is beneficial in up to 60% of patients [198], most patients relapse within 6-8 months. The mechanisms of resistance/relapse to RTX are currently unknown and it is unclear whether disease relapse is determined by B cell repopulation of the synovial membrane and whether this repopulation comes from the systemic circulation or from “escaped” clones surviving in “protected” synovial niches.

Overall, the above evidence demonstrates that an antigen-dependent autoimmune response takes place locally and triggers a self-sustained proliferation of B cells and local autoantibody production in the absence of re-circulating immune cells.

However, the proportion of synovial autoreactive B cells towards a particular antigen and the exact nature of the antigens driving the humoral autoimmune response within the RA joints in the presence of ELS are unknown and their clarification would provide novel essential information on the aetiology and pathogenesis of RA.

In order to address the above questions, I analysed 139 Ig VH gene sequences and generated 66 complete recombinant IgG, IgA and IgM monoclonal antibodies with matching H + L chains from single CD19+ synovial B cells FACS-sorted from the synovium of RA patients with functional ELS.

To confirm previous data, I did not observe a skewed distribution of the V gene usage (H + L) in the synovial B cells compared to the distribution of the V families in healthy individuals.

Interestingly, several synovial B cells with an identical rearrangement and an identical pattern of somatic mutations were found within the RA synovium which is an indication of proliferating B cell clones within the RA synovial tissue. Of relevance, I also observed highly mutated and clonally related synovial B cells which could suggest that within the inflamed synovial tissue there is a constant activation and differentiation of B cells and that this activation is antigen-driven. This hypothesis has long been suggested by previous studies performing analysis of the V-gene repertoire showing that a significant proportion of B lymphocytes accumulating within the synovial membrane of RA patients display an oligloclonal repertoire with highly mutated V regions, compatible with a local antigen driven reaction [156, 157].

However, the novelty of the experimental approach employed during my PhD is that it combines sequence analysis with recombinant monoclonal antibodies generation, allowing not only lineage tree and clonal relationship analysis, but also the identification of the antigenic specificity of lesional synovial B cells.

In particular, the fine specificity of the antigen(s) recognised by synovial B cells is unknown and it would be extremely relevant to clarify if dominant antigens are driving the activation and proliferation of B cells directly at the site of inflammation, within the same joint and across the synovium of different RA patients.

As first screening I started to evaluate the reactivity towards citrullinated antigens since anti-citrullinated protein/peptide antibodies (ACPA) are an important diagnostic marker for RA and could be pathogenic for this autoimmune disease. Among all the clones analysed, 3 monoclonal antibodies showed a positive reactivity in the anti-CCP ELISA test and further 5 clones displayed a low level immunoreactivity, although further analysis should be done in order to confirm this reactivity by testing the antibodies on specific citrullinated proteins (or derived peptides) associated with RA such as vimentin, fibrinogen, alpha-enolase, etc. Very interestingly, one of the 3 clones resulted positive in the anti-CCP ELISA test (RA2 IgG80) was found to be clonally related with another clone within the RA synovium of one patient and showed to have a share CDR3 with clones isolated from the RA synovium of a different patient. These results, if confirmed in more refined studies, could identify specific CDR3 regions shared among different RA patients responsible for the binding of B cells to citrullinated proteins (similarly to the HLADRQ “shared epitope”).

I now aim to fully characterise the fine reactivity of these recombinant antibodies towards a large panel of RA-specific and non-specific (auto)antigens using a well characterised protein array platform which has been developed by Prof William H Robinson at Stanford University (see Future plan, Chapter 8) [121]. If successful, this novel approach will allow the elucidation of the mechanisms triggering humoral autoimmunity in situ and could shed novel light into the aetiology and/or pathogenesis of RA. Moreover, the identification of relevant synovial autoantigens and distinct antibody profiles in different RA patients could allow i) improving the accuracy of the diagnosis in the early stages of the disease, ii) stratifying patients in different disease subsets and iii) paving the way for tailored vaccination strategies based on the main autoantigens actively triggering autoimmunity within the RA joints.

Chapter 8 | Future plans

8 | Future plans

8.1 Future plan: Rheumatoid arthritis.

The number of potential antigens in RA is extensive and to date there is no direct evidence of specific RA antigens driving the immune response within the RA joints. Together with the classical rheumatoid factor and anti-citrullinated peptide/proteins antibodies (ACPA) [100], other potential autoantigens include cartilage-specific proteins such as type II collagen, human cartilage gp39 (or CD40 ligand), proteoglycans and aggrecans. Among the non-articular antigens, circulating autoantibodies against glucose-6-phosphate isomerase, heterogeneous nuclear ribonucleoprotein A2/B1 and heat shock proteins have been described [121]. Additionally, it may be very well possible that a certain number of unknown endogenous and exogenous antigens are the target of an (auto)immune response within the RA synovium.

Thus, my short-term future plan in the context of RA is the identification of the fine specificity of the immunoreactivity of synovial B cells arising within ectopic germinal centres which are now well established in their ability to support an antigen-driven differentiation and proliferation of autoreactive B cells. Elucidation of the mechanisms triggering humoral autoimmunity in situ could shed novel light into the aetiology and/or pathogenesis of RA and could be used for disease stratification and prognosis in order to identify early-stage RA patients, as well as to classify responders and non-responders to anti-rheumatic therapies [199].

Recent advances in proteomic technologies have enabled large-scale analysis, parallel measurements of several proteins using small quantities of biological fluids. In

particular, the introduction of antigen microarray has been invaluable to profile autoantibodies specificities in patients with autoimmune diseases [121, 200]. Furthermore, the antigen microarrays overcome several limitations of the routine assays used in clinical laboratories to measure autoantibodies which require large quantities of biological samples and allow the detection only of one antibody at a time [200].

The antigen microarrays commonly used are the solid-phase, planar arrays. In the planar microarray the antigens are immobilized on nitrocellulose membranes or onto poly-L-lysine-coated microscope glass slides in an ordered array. The array is then probed with samples of interest (e.g. sera, plasma or culture supernatants), thus a secondary antibody conjugated either to an enzyme or to a fluorochrome is added to the array in order to visualize and quantify the antigen-specific antibodies. Hämmerle and colleagues were the first group to develop an antigen microarray to detect autoantibodies in biological fluids [201]. Afterwards, several antigen microarrays have been developed and adapted to specific autoimmune diseases. Robinson and colleagues have modified the antigen microarrays with specific antigens found in the synovial tissue of the joint for profiling of RA, with myelin peptides for profiling of multiple sclerosis, or with nuclear antigens for profiling of SLE. The advantage of this approach is the possibility to test post-translationally modified antigens as opposed to native proteins which are frequently poorly immunogenic in vivo. Thus:

1) the short-term plan is to take advantage of the recombinant monoclonal antibodies generated from B cells isolated from the RA synovium and characterize the fine specificities of the (auto)reactivity profile of these monoclonal antibodies using the

antigen microarray platform which has been developed by the laboratory of William Robinson at Stanford University (**Figure 8.1**) [121]. In particular, Robinson's lab has developed a synovial array which is characterized by >200 peptides and proteins spotted on a 1536-feature array. This panel of antigens which are candidate autoantigens in RA include native and in vitro citrullinated keratin, fillagrin, vimentin, and fibrinogen, as well as heat-shock proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs) A2/B1, glucose-6-phosphate isomerase, human cartilage gp39 and many others [121].

For the purpose of my project, the RA antigen microarray will be probed with the purified recombinant monoclonal antibodies that I have generated. This platform is extremely powerful since the current assays such ELISA, western-blot analysis and radioimmunoassay do not allow a large-scale multiplex characterization of autoantibody response and most importantly they require large amount of antibodies or patient samples. A collaboration has been established with Prof Robinson's lab and I will spend some times at Stanford in order to carry out this part of the project.

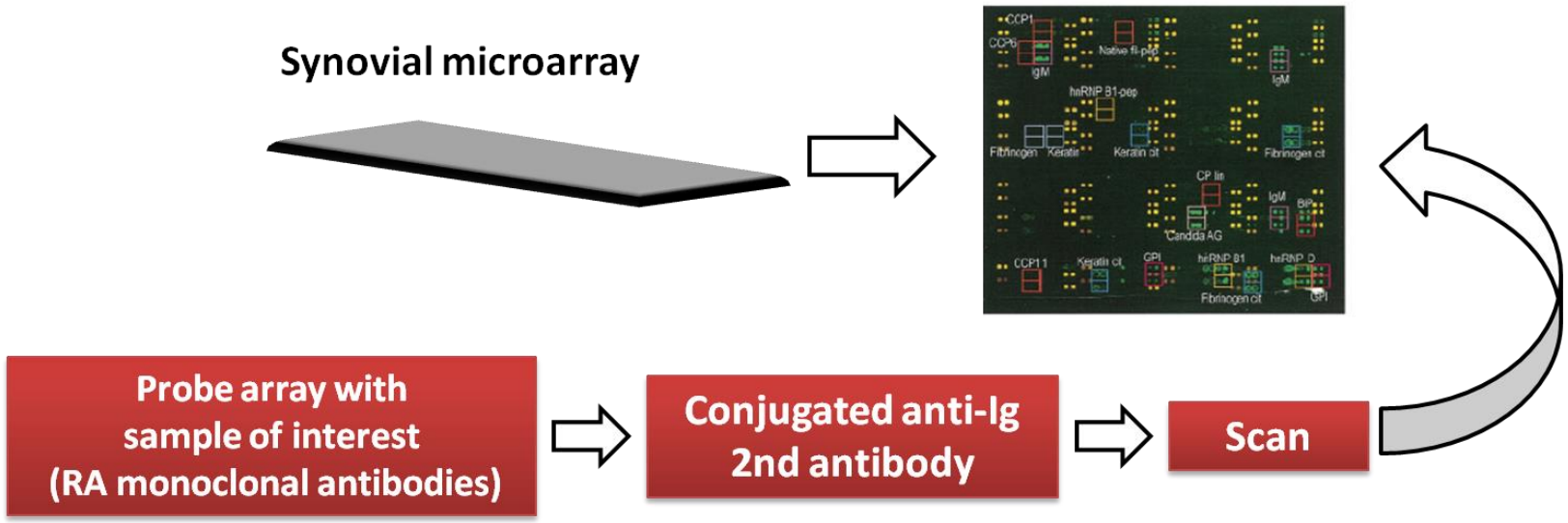

Figure 8.1 Antigen array for antibody profiling.

Synovial antigen microarray containing peptides and proteins representing candidate RA autoantigens are used to profile autoantibodies in samples of interest (e.g. sera from RA patients or recombinant monoclonal antibodies) and to identify autoantibody biosignatures correlated with clinical features that can predict development of more severe arthritis [121].

2) in the longer term, I am planning to **i)** develop synovial cDNA libraries as an alternative approach to identify potential novel autoantigens. This work is planned in collaboration with the University of Novara, Italy, where synovial cDNA libraries are being developed. The advantage of this approach is the possibility of a high throughput and unbiased analysis of the antigenic specificities with the potential for the identification of novel autoantigens. The disadvantage, as mentioned before, is the limitation of testing the antibodies on native antigens, possibly limiting antigenicity.

The identified autoantigens, either by protein arrays or synovial cDNA libraries will be then assessed on larger cohorts of RA patients and controls in order to assess specificity for RA and stratify patients having similar autoantigen responses towards "dominant" antigens. Additionally, I am planning **ii)** to expand the analysis of CDR3 sequences shared by a large number of clones in different patients as this approach could allow the identification of common autoreactive CDR3 signatures (i.e. towards citrullinated antigens), which would provide a clue on how the humoral autoimmune response towards a particular antigen is shaped within the synovial microenvironment.

Finally, a foreseeable future step of my research is **iii)** testing the recombinant monoclonal antibodies with autoreactive profile for pathogenicity. For this purpose, I will challenge my recombinant antibodies in animal models of arthritis such as the collagen-induced arthritis model where it has been shown that ACPA can worsen the disease [202].

8.2 Future plan: Sjögren's syndrome.

The obvious direction of my research post-PhD in SS will be mirroring the work done on the RA joints and translate this into the analysis of lesional B cells in the salivary glands of SS patients.

Similarly to RA, I will focus on the 30% of patients who develops functional germinal centres in the salivary glands, which accounts to around 30% of SS patients. As discussed throughout this thesis, ELS in the SS salivary glands are fully functional and support the differentiation of autoreactive B cells towards plasma cells producing high affinity autoantibodies.

Here the experimental approach will be similar to RA, and I will focus primarily on SS patients developing swelling of the major salivary glands (i.e. parotids and submandibular) as they will provide larger material for B cell separation. Additionally, this would be very relevant clinically, as patients with salivary gland swelling are more prone to develop lymphoma at a later stage of disease.

Thus, in this context, I have already collected and store B cells isolated from parotid glands of SS patients which are ready to be used for recombinant monoclonal antibody production. The aims of my future plans in SS mirror those listed in the context of RA and can be summarised as follows:

1) analysis of the autoreactivity profile of lesional B cells with identification of novel autoantigen specificities and/or fine mapping of dominant epitopes driving the autoimmune response in the salivary glands.

2) assessment of pathogenicity of lesional B cells. In particular I will focus on the reactivity towards the muscarinic M3R, which is the most promising target of direct autoantibody-mediated exocrine dysfunction in SS patients. Upon successful identification of anti-M3R antibodies (in collaboration with Prof Tsumida in Japan), I will then test their capacity to induce salivary hypofunction in rodents.

3) identification of the antigenic specificities of malignant B cells in MALT lymphomas of the salivary glands. This is a main priority of my work as it has enormous clinical relevance. Malignant MALT-L B cells have the unique characteristic of originating from autoreactive B cells with around 40% of lymphomatous parotid B cells displaying rheumatoid factor reactivity [203]. These lymphomas arise as a result of a continuous antigen driven immune response in the glands leading to the accumulation of genetic instability resulting in oligo- and then monoclonal proliferation and escape of malignant clones. This process is believed to take place in ectopic germinal centres which have been shown to be independent risk factor for lymphoma development in SS patients [73, 181]. Thus, I will use my experimental approach and investigate the autoantigenic profile of lymphomatous B cells, in particular focusing on the putative 60% of clones which do not display RF reactivity. The clarification of such immunoreactivity could allow a better understanding of the process leading to lymphoma development as well as potentially the identification at an early stage of SS patients at higher risk of B cell lymphoma.

Appendix

Solutions and buffers

- **1X PBS (Lonza):** 144 mg/L KH_2PO_4, 9,000 mg/L NaCl, 795 mg/L Na_2HPO_4 without calcium and magnesium (pH: 7.3-7.5).
- **10X TBE (Gibco):** 1M Tris, 0.9 M Boric Acid, 0.01 M EDTA.
- **10X TBS (pH 7.5):** 9.7 g Trizma Base (Sigma), 66.1 g Trizma Hydrochloride (Sigma), 90 g Sodium Chloride (Sigma) dissolved in 1 L of distilled water.
- **Ultrapure 0.5 M EDTA pH 8.0 (Gibco):** sterile filtered solution prepared by dissolving $Na_2EDTA•2H_2O$ in distilled, deionized water and adjusting the pH to 8.0 with sodium hydroxide.
- **Histopaque – 1077 (Sigma):** solution of polysucrose 57 g/L and sodium diatrizoate 90 g/L, adjusted to a density of 1.077 g/ml (pH: 8.8-9.0).
- **Trypan Blue solution 0.4% (Sigma):** prepared in 0.81% sodium chloride and 0.06% potassium phosphate, dibasic. Used at a concentration of 0.2%, diluted in PBS.
- **Sodium azide (NaN_3) 5%:** 2.5 g NaN_3 (Sigma) dissolved in 50 ml of distilled water.
- **FACS buffer staining solution:** 1X PBS supplemented with 0.5% fetal bovine serum (FBS) (Gibco).
- **Nutridoma SP (Roche):** serum-free medium supplement composed of albumin, insulin, transferrin, and other defined organic and inorganic compounds. Nutridoma contains no other growth factors, mitogens, hormones, or sterols.
- **Complete culture medium (293T):** Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 **(**DMEM/F-12) with high concentration of glucose (GlutaMAX) (Invitrogen) supplemented with 10% heat-inactivated FBS (Gibco), 100 μg/ml streptomycin, 100

U/ml penicillin G and 0.25 μg amphotericin (all from Invitrogen), and 500 μg/ml Geneticin (Invitrogen).

- **"Nutridoma" medium (293T transfection):** Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 **(**DMEM/F-12) with high concentration of glucose (GlutaMAX) (Invitrogen) supplemented with 1% Nutridoma SP (Roche), 100 μg/ml streptomycin, 100 U/ml penicillin G and 0.25 μg amphotericin (all from Invitrogen).
- **Lysogeny broth (LB) Agar plates:** 10 g BactoTryptone, 5g Yeast Extract, 15 g Agar, 5 g NaCl are added to 1 L distilled water. The solution is autoclaved and allowed to cool down below 50 °C before adding the Ampicillin (Sigma) at a concentration of 100 μg/ml. Mix thoroughly before pouring into petri dishes.
- **Terrific broth (TB) medium (1L):** 12 g BactoTryptone, 24 g Yeast Extract, 4 ml Glycerol are added to 900 ml distilled water. The solution is sterilized by autoclaving and allowed to cool down to room temperature. Finally 100 ml of a filtered sterilized solution made with 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 is added to reach a final volume of 1L.
- **0.1 M Glycine (pH 3):** 3.8 g glycine dissolved in 500 ml distilled water.
- **1M Tris-HCl (pH 8.0):** 60.6 g Trizma Base (Sigma) dissolved in 500 ml distilled water.

Bacteria strains and human cell lines

- **Genotype competent cells DH10B (NEB):** *araD139* Δ(*ara-leu*)7697 *fhuA lacX74 galK* (φ80 Δ(*lacZ*)*M15*) *mcrA galU recA1 endA1 nupG rpsL* (Str^R) Δ(*mrr-hsdRMS-mcrBC*).
- **Human embryonic Kidney (HEK) 293T:** human cell line derived from embryonic kidney cells transformed with adenovirus 5 DNA. 293T is a highly transfectable derivative of the 293 cell line containing the SV40 T-antigen.

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